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DRAFT

MEMORANDUM

Mtg w/ Bill B
Chris / Aubrey 6/17/02

To: Chris Weis

cc: Jim Christiansen

From: Alison Van Gorp, Bill Brattin

Subj: Design of Animal Inhalation Study for Libby Asbestos

Date: June 6, 2002

$1.2M ?

Morgantown
→ NIOSH!
Vince Castranova

- Source material rep. of what Libby people exposed to. Key!
- Academic institution COI/experience/likely not U of M
- chrysotile "control" or test pop Key!
- USGS - Dirty 30 -
- Generation of Fiber

Chris:

As you requested, SRC has been working to design an animal study on the inhalation toxicity of asbestos material from the Libby mine. To begin this process, we retrieved and reviewed a number of published studies on the inhalation toxicity of asbestos in animals. These are listed in the reference list, and are summarized in Table 1. Based on our review of these studies, we have drawn the following main conclusions:

Test Species

In the literature, the rat was the most commonly studied animal in asbestos inhalation studies (23 of 29 inhalation studies reviewed). Additionally, based on lung dosimetry and histopathology, it is believed that the rat most closely predicts a human response to inhaled fibers (Maxim and McConnell 2001). On this basis, we propose that the study should be performed using rats. However, the rat model must be interpreted with caution as some studies have suggested that humans may be up to 300 times more sensitive to tumor risk from asbestos inhalation than rats (Rodelsperger and Woitowitz 1995).

Exposure Method

Several different dust generator and exposure chamber combinations have been described in the literature. The exposure chambers ranged from whole room "walk-in" chambers (e.g. Gross and de Treville 1967, Reeves et al. 1974) to chambers containing the living areas for the animals (Timbrell et al. 1970) to an array of small tubes, each holding one animal during the exposure period (Cannon et al. 1983, Bernstein et al. 1994). The larger exposure chambers have inherent problems in that fibers fall onto the bedding and food of the animal and can be re-dispersed into the air when animals disturb the substrate. Additionally, these larger chambers have uneven fiber concentrations in various areas of the chamber that can result in highly variable exposures between animals. Consequently, the nose-only exposure chamber was developed. This system involves an array of clear plastic tubes (each of which houses an animal during the exposure period) around a central cylinder through which the fiber aerosol is delivered to each animal. Due to concerns that this system may also result in uneven exposures between animals, Cannon et al. devised a flow-past system in which animals inhale the fiber aerosol from a central tube and exhale into a separate exhaust tube. This system ensures that each animal breathes in the aerosol at its full concentration and it is not diluted by exhaled air from other animals. Bernstein et al. (1994) combined Cannon's flow-past nose-only exposure chamber with a dust generator of their own design. This dust generation system creates an aerosol with a high percentage of unbroken fibers and little non-fibrous dust.

Each exposure apparatus can house up to 160 individual animals, and a different apparatus is required for each dose group.

Cumulative Exposure

Effects of asbestos are likely to be related to the cumulative lung burden of durable fibers. Lung burden is a function of both exposure duration and exposure concentration (in air). Thus, a convenient way to characterize exposure is cumulative exposure.

$$CE = ED*EC$$

where:

CE = Cumulative Exposure (mg/m^3-hrs or f/cc-hrs)
ED = Exposure Duration (hrs)
EC = Exposure Concentration (mg/m^3 or f/cc)

In the literature, exposure typically occurs for 6 hours per day, 5 days per week, for anywhere from one week to two years. The average study duration in the articles we reviewed is slightly under 8 months. However, some studies used very short exposure periods of 1 hour (Barry et al. 1983) or 1 (7-hour) day (Wagner et al. 1974). The most common exposure level used in animal studies to date is 10 mg/m^3, although some studies have used exposure less than 1 mg/m^3. Assuming an average fiber length of 5 um, an average fiber thickness of 0.5 um and an average density of 2.5 g/cc, a concentration of 10 mg/m^3 corresponds to about 3,400 f/cc.

Table 1 summarizes the cumulative exposures for each of the literature studies were reviewed. As seen, most CE values ranged from 9E+00 to 2E+05 mg-hrs/m^3. In cases where tumors were observed, CE values ranged from 1E+03 to 2E+05 mg-hrs/m^3. In fact, tumors were observed in 48% of the exposures with CE values greater than 1E+03 mg-hrs/m^3.

Endpoints

There are a wide variety of different endpoints that have been measured in animal studies of asbestos toxicity. These are summarized below.

Immune Cell Density in the Lung

This method requires the rinsing of the lung with a solution which is then captured and centrifuged (lavage). The resulting cells are stained and then examined for macrophages, neutrophils, lymphocytes, monocytes, binucleate macrophages, and multinucleate macrophages. Three of the studies examined in this review utilized bronchioalveolar lavage (Rihn et al. 1996, Rihn et al. 2000, Donaldson et al. 1988). All three studies reported increased levels of these immune-response cells after exposure to asbestos via inhalation.

Histopathology of the Lung

Nearly all of the inhalation studies we reviewed included a histological examination. Similar methodology was employed by the researchers in preparing the lung for examination. The lungs (or lung) are removed and fixed by inflation with Karnovsky's fixitive. The lung is sectioned horizontally for a routine histological exam. Additionally, any grossly visible lesions are sectioned for examination. After the lung tissue was prepared, it was examined for the following abnormalities:

- Macrophage Infiltration
- Alveolar bronchiolization
- Microgranuloma
- Giant Cells
- Interstitial fibrosis
- Mesothelial hyperplasia
- Pleural fibrosis and collagen deposition

These histological endpoints are often graded on severity so that quantitative comparisons between study groups are possible. Commonly used grading scales are the Wagner grade and a pathology grading scale developed by McConnell et al. (1984) for pulmonary cellular change and fibrosis. Additionally, grading scales have been developed for the other endpoints, ranking the endpoint tissues from normal (0) to widespread and severe (5) (Hesterberg et al 1997, McConnell et al 1995, Mast et al.1995).

Carcinogenisis

As a part of the histopathological exam, the following forms of cancer were found in some of the inhalation studies reviewed.

- Mesothelioma
- Carcinoma
- Adenoma

Thirteen studies saw increased rates of cancer in exposed animals. For example, Wagner et al. (1974) reported increases in adenomatosis, adenocarcinoma and squamous carcinoma after exposures to amosite, anthophyllite, crocidilite, and chrysotile asbestos. Furthermore, increased duration of exposure produced higher rates of these cancers.

Biochemistry

- Cell Proliferation (BrdU marker)

  Two studies we reviewed used BrdU as a marker for cell proliferation and DNA synthesis (Coin et al. 1996, BeruBe et al. 1996). In the Coin et al. study, sections of lung were stained with BrdU after the animals were sacrificed. Alternatively, in the BeruBe et al. study, the animals were injected intraperitoneally with BrdU 24- to 48-hours prior to sacrifice. Both studies showed evidence of enhanced cell proliferation/DNA synthesis after exposures to crysotile asbestos.

- Mutagenisis (transgenic mice)

  Rihn et al. (2000) investigated mutagenicity in lung DNA after exposure to crocidilite. Transgenic mice carrying the *lacI* reporter gene were exposed to crocidilite for 5 days, after which the endpoints were monitored at 1, 4 and 12 weeks into the recovery period. In the fourth week after exposure there was a significant increase in mutant frequency between exposed animals and controls. The induction factor (the ratio of mutations in exposed mice to those in control mice) was 1.96, indicating a nearly two-fold increase in the mutation rate after exposure to crocidilite.

Lung Burden and Lung Fiber Size Distribution

The lung burden is dependant upon both the exposure concentration and duration and is a measure of the total number of fibers in the lung at the time of sacrifice. All of the studies reviewed used essentially the same method for determining lung burdens and fiber distributions. One lung (or a part of one lung) was dried, weighed, low temperature ashed, and suspended in pure water. The suspension was then filtered to capture the fibers. The filter was applied to a grid and examined using either TEM or SEM techniques. Fibers were enumerated and measured. Lung burdens are presented in several ways (e.g. fibers/lung, fibers/animal, fibers/mg dry lung, etc.), but the fibers/mg dry lung is most useful for our purposes as it can be used in interspecies comparisons. Fiber size distributions are created from the length and width measurements recorded during the counting procedure. They are presented as either a 3-D figure showing the frequency of fibers in certain length-width categories or as a table presenting the number of fibers falling into specific length or width categories.

Biopersistance of Fibers

This endpoint is related to the fiber size distribution. It requires at least two study groups, one that is sacrificed immediately after the exposure period, and one that is sacrificed after some recovery period (usually 6 to 12 months). A comparison can then be made between the fiber size distributions in the lungs of the two groups. Commonly, most of the short fibers are cleared from the lung during the recovery period, whereas a certain fraction of the long fibers remain in the lung. For example, Hesterberg et al. (1998) found that 90 days after termination of exposure (2yrs at 10 mg/m^3) short fibers had decreased by 90% and long fibers had decreased by 65%. Moreover, after 12 months from termination of exposure there were little or no further reductions in amosite fibers in either length category. Thus, it appears that all fibers that can be cleared are done so in a relatively short period and the remaining fibers will be retained in the lung. Also of importance, it has been noted that there is an apparent correlation between the biopersistance of fibers longer than 20 um and the onset of pulmonary pathogenisis (Hesterberg et al. 1998).

For this study we recommend analysis of the following endpoints: histology, cancer, lung burden and fiber distribution.

Recommended Study Design

After reviewing the literature we have devised four alternative study designs. All four studies involve exposure of rats (usually 40 per group) at four different exposure levels (10, 1.0, 0.1, and 0.0 mg/m^3)

for varying time patterns of exposure and observation. The mass of material needed to support these materials in on the order on 20 g (see Attachment A).

The four options, as well as their pros and cons, are outlined below.

Option 1

See Figure 1, Table 1 for study design details.

Pros:
- Relatively inexpensive

Cons:
- shorter exposure periods may not result in significant cancer findings
- no comparison to animals exposed with a recovery period

Option 2

See Figure 2, Table 2 for study design details.

Pros:
- comparison between animals with and without a recovery period after exposure

Cons:
- shorter exposure periods may not result in significant cancer findings
- somewhat more costly

Option 3

See Figure 3, Table 3 for study design details.

Pros:

- comparison between animals with and without a recovery period after exposure
- longer exposures should result in significant cancer findings

Cons:
- relatively expensive

Option 4

See Figure 4, Table 4 for study design details.

Pros:
- long recovery periods allow for analysis of long-term fiber clearance rates

Cons:
- shorter exposure periods may not result in significant cancer findings
- somewhat more costly

Comparison of Estimated Cumulative Exposures and Lung Burdens

Table 2 compares the range of cumulative doses that would be expected based on the four alternative experimental designs, in comparison to the range of exposures that might be expected in Libby. As seen, the low end of the animal exposure overlaps with the expected range of human exposures, increasing the relevance of the animal studies.

ATTACHMENT A

Mass of Libby Amphibole Material Required for Study Design 2

Flow rate = 10 L/min/exposure chamber

Groups 1 - 3: 0.01 mg/m³

$$\frac{10L}{\min}\cdot\frac{1m3}{1000L}\cdot\frac{0.01mg}{m3}\cdot\frac{60\min}{hr}\cdot\frac{6hr}{day}\cdot\frac{5days}{week}\cdot 26weeks = 4.68mg = 0.005g\cdot 3groups = 0.014g$$

Groups 4 - 6: 0.1 mg/m³

$$\frac{10L}{\min}\cdot\frac{1m3}{1000L}\cdot\frac{0.1mg}{m3}\cdot\frac{60\min}{hr}\cdot\frac{6hr}{day}\cdot\frac{5days}{week}\cdot 26weeks = 46.8mg = 0.047g\cdot 3groups = 0.140g$$

Groups 7 - 9: 1.0 mg/m³

$$\frac{10L}{\min}\cdot\frac{1m3}{1000L}\cdot\frac{1mg}{m3}\cdot\frac{60\min}{hr}\cdot\frac{6hr}{day}\cdot\frac{5days}{week}\cdot 26weeks = 468mg = 0.468g\cdot 3groups = 1.404g$$

Groups 10 - 12: 10 mg/m³

$$\frac{10L}{\min}\cdot\frac{1m3}{1000L}\cdot\frac{10mg}{m3}\cdot\frac{60\min}{hr}\cdot\frac{6hr}{day}\cdot\frac{5days}{week}\cdot 26weeks = 4{,}680mg = 4.680g\cdot 3groups = 14.040g$$

Total LA Required = 9.4 + 0.94 + 0.09 = 15.6g

Table 1: Summary of Animal Inhalation Studies

Study	Animal	Asbestos Type	Exposure Level (mg/m3)	hrs/day	days/wk	weeks	Total Exposure Time (hours)	Cumulative Exposure (mg-hrs/m3)	Endpoints	Histo	Biochem	Cancer
Bernstein et al. 1994	Rat	Crocidolite	10	6	5	1	30	300	Size Dist, lung burden, chem composition of fib			
Hesterberg et al. 1998a	Rat	Amosite	17	6	5	1	30	510	Size dist, lung burden			
Hesterberg et al. 1996	Rat	Crocidolite	10	6	5	1	30	300	Size dist, lung burden			
BeruBe et al. 1996	Rat	Chrysotile, Cr	8	6	5	1	30	240	BioChem, Histo, lung burden	+	+	
BeruBe et al. 1996	Rat	Chrysotile, Cr	8	6	5	4	120	960	BioChem, Histo, lung burden	+	+	
Hesterberg et al. 1997	Hamster	Amosite	0.8	6	5	13	390	312	Histo, Lung Burden, Size Dist, Cancer			
Hesterberg et al. 1997	Hamster	Amosite	0.8	6	5	26	780	624	Histo, Lung Burden, Size Dist, Cancer			
Hesterberg et al. 1997	Hamster	Amosite	0.8	6	5	52	1560	1248	Histo, Lung Burden, Size Dist, Cancer			+
Hesterberg et al. 1997	Hamster	Amosite	3.7	6	5	13	390	1443	Histo, Lung Burden, Size Dist, Cancer			
Hesterberg et al. 1997	Hamster	Amosite	3.7	6	5	26	780	2886	Histo, Lung Burden, Size Dist, Cancer			
Hesterberg et al. 1997	Hamster	Amosite	3.7	6	5	52	1560	5772	Histo, Lung Burden, Size Dist, Cancer			+
Hesterberg et al. 1997	Hamster	Amosite	7.3	6	5	13	390	2847	Histo, Lung Burden, Size Dist, Cancer			
Hesterberg et al. 1997	Hamster	Amosite	7.3	6	5	26	780	5694	Histo, Lung Burden, Size Dist, Cancer			
Hesterberg et al. 1997	Hamster	Amosite	7.3	6	5	52	1560	11388	Histo, Lung Burden, Size Dist, Cancer			+
Hesterberg et al. 1998b	Rat	Chrysotile	10	6	5	104	3120	31200	histo, lung burden, size dist	+		+
Mast et al. 1995	Rat	Chrysotile	10	6	5	13	390	3900	Histo, Lung Burden, Size Dist, Cancer			+
Mast et al. 1995	Rat	Chrysotile	10	6	5	26	780	7800	Histo, Lung Burden, Size Dist, Cancer			+
Mast et al. 1995	Rat	Chrysotile	10	6	5	52	1560	15600	Histo, Lung Burden, Size Dist, Cancer			+
Mast et al. 1995	Rat	Chrysotile	10	6	5	65	1950	19500	Histo, Lung Burden, Size Dist, Cancer			+
Mast et al. 1995	Rat	Chrysotile	10	6	5	78	2340	23400	Histo, Lung Burden, Size Dist, Cancer			+
Mast et al. 1995	Rat	Chrysotile	10	6	5	104	3120	31200	Histo, Lung Burden, Size Dist, Cancer			+
McConnell et al. 1995	Hamster	Chrysotile	10	6	5	13	390	3900	Histo, Lung Burden, Cancer			-
McConnell et al. 1995	Hamster	Chrysotile	10	6	5	26	780	7800	Histo, Lung Burden, Cancer			-
McConnell et al. 1995	Hamster	Chrysotile	10	6	5	39	1170	11700	Histo, Lung Burden, Cancer			-
McConnell et al. 1995	Hamster	Chrysotile	10	6	5	52	1560	15600	Histo, Lung Burden, Cancer			-
McConnell et al. 1995	Hamster	Chrysotile	10	6	5	78	2340	23400	Histo, Lung Burden, Cancer			-
Platek et al. 1985	Rat, Monkey	Chrysotile	1	7	5	78	2730	2730	Histo, Lung Burden, Size Dist, Cancer			-
Rihn et al. 2000	Mice	Crocidolite	5.75	6	5	1	30	172.5	BioChem, Histo, lung burden	+		
Rihn et al. 1996	Mice	Crocidolite	13.6	6	5	1	30	408	Histo, Lung Burden	+		
Botham and Holt 1972	Rat, Guinea	Crocidolite	not reported	20	5	4	400	#VALUE!	histo	+		
Gross et al. 1967	Rat	Chrysotile	86	6	5	62	1860	159960	histo, cancer			+
Wagner et al. 1974	Rat	Amosite, Anth	11.0989011	7	5	13	455	5050	histo, cancer			+
Wagner et al. 1974	Rat	Amosite, Anth	11	7	1	1	7	77	histo, cancer			
Wagner et al. 1974	Rat	Amosite, Anth	9.307692308	7	5	26	910	8470	histo, cancer			+
Wagner et al. 1974	Rat	Amosite, Anth	9.395604396	7	5	52	1820	17100	histo, cancer			+
Wagner et al. 1974	Rat	Amosite, Anth	9.175824176	7	5	104	3640	33400	histo, cancer			+
Hiett 1978	Guinea Pig	Chrysotile, Am							Functional, histo			
Barry et al. 1983	Rat	Chrysotile	9.06	1	1	1	1	9.06	histo			
Barry et al. 1983	Rat	Chrysotile	9.06	7	5	1	35	317.1	histo			
Barry et al. 1983	Rat	Chrysotile	9.06	7	5	13	455	4122.3	histo			
Sjostrand et al. 1989	Guinea Pig	Amosite	49	2	5	3	30	1470	histo			
Sjostrand et al. 1989	Guinea Pig	Amosite	49	2	5	6	60	2940	histo			
Coin et al. 1996	Rat	Chrysotile	10	3	3	1	9	90	BioChem, Histo, Size dist	+	+	
Coin et al. 1996	Rat	Chrysotile	10	5	3	1	15	150	BioChem, Histo, Size dist	+	+	
Wehner et al. 1975	Hamster	Chrysotile	23	7	5	48	1680	38640	histo, Cancer			+
Reeves et al. 1971	Rat, Rabbit,	Amosite, Croc	48	4	4	4	64	3072	histo, Cancer			+
Reeves et al. 1971	Rat, Rabbit,	Amosite, Croc	48	4	4	13	208	9984	histo, Cancer			+
Reeves et al. 1971	Rat, Rabbit,	Amosite, Croc	48	4	4	26	416	19968	histo, Cancer			+
Reeves et al. 1971	Rat, Rabbit,	Amosite, Croc	48	4	4	52	832	39936	histo, Cancer			+
Reeves et al. 1971	Rat, Rabbit,	Amosite, Croc	48	4	4	104	1664	79872	histo, Cancer			+
Reeves et al. 1974	Rat, Rabbit,	Amosite, Croc	49	4	4	104	1664	81536	histo, Cancer			+

+ = Effect
– = No effect

Study	Animal	Asbestos Type	Exposure Level (mg/m3)	hrs/day	days/wk	weeks	Total Exposure Time (hours)	Cumulative Exposure (mg-hrs/m3)	Endpoints	Histo	Biochem	Cancer
Davis et al. 1991	Rat	Amosite, Chry	10	5	5	52	1300	13000	histo, lung burden, Cancer			+
Davis et al. 1985	Rat	Tremolite, Buc	10	7	5	52	1820	18200	histo, lung burden, Cancer			+
Davis et al. 1988	Rat	Chrysotile	10	7	5	52	1820	18200	Histo, lung burden, Biopersistance, Cancer			+
Donaldson et al. 1988	Rat	Chrysotile	10	7	5	15	525	5250	Histo, macrophage activity assay	+		
Davis and Jones 1988	Rat	Chrysotile	10	7	5	52	1820	18200	Histo, lung burden, Cancer			+
Davis et al. 1986a	Rat	Chrysotile	5	7	5	52	1820	9100	Histo, lung burden, Cancer			+
Davis et al. 1986b	Rat	Amosite	10	7	5	52	1820	18200	Histo, lung burden, Cancer	+		+
Gross and deTreville 1967	Rat	Chrysotile	86	6	5	1	30	2580	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	86	6	5	2	60	5160	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	86	6	5	4	120	10320	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	86	6	5	8	240	20640	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	86	6	5	17	510	43860	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	86	6	5	35	1050	90300	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	20	6	5	4	120	2400	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	20	6	5	8	240	4800	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	20	6	5	17	510	10200	histo	+		
Gross and deTreville 1967	Rat	Chrysotile	20	6	5	26	780	15600	histo	+		
Gross and deTreville 1967	Hamster	Chrysotile	20	6	5	4	120	2400	histo	+		
Gross and deTreville 1967	Hamster	Chrysotile	20	6	5	8	240	4800	histo	+		
Gross and deTreville 1967	Guinea Pig	Chrysotile	86	6	5	24	720	61920	histo	+		
Gross and deTreville 1967	Guinea Pig	Chrysotile	86	6	5	13	390	33540	histo	+		
Hesterberg et al. 1998	Rat	Amosite	17	6	5	1	30	510	Size dist, lung burden			-

Table 2. Estimated Cumulative Doses

Scenario	Exposure Level	Conc f/cc	Conc mg/m3	ET hr/d	EF d/wk	EF wk/yr	ED yr	CE mg/m3-hr
Libby resident	Low	0.01	0.0000	24	7	52	40	10
	High	1	0.003	24	7	52	40	1028
Rat, Option 1&2	Low	34	0.1	6	5	4	1	12
	High	3400	10	6	5	26	1	7800
Rat, Option 3&4	Low	34	0.1	6	5	12	1	36
	High	3400	10	6	5	52	1	15600

William Brattin <brattin@syrres.com>

10/23/02 05:12 PM

To: Lori <moilanen@syrres.com>, Jim Christiansen/EPR/R8/USEPA/US@EPA, Chris Weis/NEIC/USEPA/US@EPA, amiller@osophs.dhhs.gov

cc:

Subject:

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see attached in re todays discussion on the animal study
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****
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****

Memo 10-23-02.wp(

OBJECTIVES:

1. Perform an inhalation study in animals that produces reliable dose-response and duration-response data (both short-term and long-term) on the non-cancer effects of Libby amphibole fibers on lung. The purpose of these data is to help evaluate the potential for non-cancer effects in humans exposed to Libby amphiboles by inhalation., and to establish a site-specific RfC.

2. Obtain data on the relative potency of Libby amphiboles compared to one or more well characterized fibrous materials.

PROPOSED STUDY DESIGN

Dose Group	Dose material	Dose level	Exposure Duration	Total N	Sacrifice Times							
					1 wk	2 wk	4 wk	8 wk	16 wk	24 wk	52 wk	78 wk
1	None	--	24 wk	40	5	5	5	5	5	5	5	5
2	LA	A	24 wk	40	5	5	5	5	5	5	5	5
3		B	24 wk	40	5	5	5	5	5	5	5	5
4		C	24 wk	40	5	5	5	5	5	5	5	5
5		D	24 wk	40	5	5	5	5	5	5	5	5
6	Ref Material	B	24 wk	40	5	5	5	5	5	5	5	5
7		D	24 wk	40	5	5	5	5	5	5	5	5

"Miller, Aubrey" <AMiller@osophs.dhhs.gov>

10/24/02 09:59 AM

To: 'William Brattin' <brattin@syrres.com>, Lori <moilanen@syrres.com>, Jim Christiansen/EPR/R8/USEPA/US@EPA, Chris Weis/NEIC/USEPA/US@EPA
cc:
Subject: RE: Libby animal study

Bill ....would also add to the summary:

I. points of our discussion concerning the identification and acquisition of libby amphibole to be used for the animal study.....ie..comparison of physical characteristics of USGS finely ground bulk material to the material collected on air filters...if these are not comparable we need to quickly discuss how collection of suitable material (ie, possible airborne fiber collected from mechanically agitated bulk materials)can be accomplished.........

II. Also, we need to research ASAP some of the critical issues that effect either the number of animals needed or the length of dosing (exposure time)since these parameters are critical for costing out the research....such information would include: 1)the variability of non-cancer endpoints observed in the selected species at a given dose, 2) the length of time needed to observe our non-cancer endpoint of interest (ie. pulmonary fibrosis), 3)also identification of any other health endpoints we wish to evaluate....

Regards, Aubrey

PS:

If possible I would like to obtain a copy of the articles cited at the end of the draft design you sent me last week.

-----Original Message-----
From: William Brattin [mailto:brattin@syrres.com]
Sent: Wednesday, October 23, 2002 5:12 PM
To: Lori; Jim Christiansen; Chris Weis; Miller, Aubrey
Subject:

see attached in re todays discussion on the animal study

****
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15 November 2002

Dr. Lori Moilanen
Senior Toxicologist
Syrres Corp.

Lori,

Per your request, following are the methods and endpoints of most import in evaluating the pathology from rodent inhalation studies, specifically the rat. The rat lung consists of a single left lung and 4 lobes in the right lung. This configuration allows one to conduct several studies on the same animal, e.g. histopathology, lung fiber burden and bronchoalveolar lavage. In terms of the histopathology, the removal and handling of the lungs is critical, especially for a study such as this where nontumor pathology (fibrosis) is the endpoint of interest. My suggestions as to how to evaluate the lung are as follows:

Postmortem examination: The techniques used for the postmortem examination are particularly important. To determine a dose response for the endpoints of interest, the lungs must be handled in a standard way that allows the pathologist to "grade" the lesions as to their severity. This starts at the necropsy. The necropsies from all exposure groups and at all time points should all be conducted on the same day (at most two), preferably on a Thursday and/or Friday. This is so that the animals have had 3-4 days exposure as compared with a Monday, when they have had the weekend to recover (fiber burdens can be influenced if not done in this way). This is particularly important early in the study (I can explain the reasons for this if you wish). The animals must be euthanized by injection (preferably intraperitoneal) of an anesthetic that does not produce iatrogenic pulmonary lesions. This precludes the use of inhalation or IV anesthetics. After the animal is dead, the lungs are carefully removed at the level of the mid-trachea, are weighed and carefully examined (preferably with a dissecting microscope). One of the right lobes is tied off, weighed and frozen (this lobe is used for fiber burden). The rest of the lung is perfused with fixative via the trachea at constant pressure (30-cm water) for 2-4 hours. The trachea is then tied off and the lung is placed in with the other tissues collected at necropsy. The diaphragm can also be collected for histology and fiber burden and the rib cage for histopathology. Lung burden on the diaphragm is a fair surrogate for the types and amounts of fibers that reach the pleura.

Histology preparation: After fixation, a single standard horizontal slice of the left lung is taken at the level of the mainstem bronchus. This provides a section that allows the pathologist to examine the major and minor airways, terminal bronchioles, alveolar duct, alveoli and pleura. Histology sections are made from this slide and stained with H&E and a special stain for collagen (required to ascertain early fibrosis). Other sections of lung are taken where gross lesions are observed but are not used for grading of lesions (see below).

Histopathological examination: It is imperative that the slides from all of the animals be examined by the same pathologist using a standard technique. The pathology approach that I think works best requires examining the lungs for specific endpoints. These include macrophage response, microgranuloma formation, metaplasia at the terminal bronchiole junction (often referred to as bronchiolization), foreign body cells, and collagen deposition (evidence of fibrosis). The pleura is also evaluated for collagen deposition and mesothelial cell changes. All of these endpoints are graded on a scale from 0-4 (0=normal, 1=minimal, 2=mild, 3=moderate, 4=marked). Once fibrosis is observed it should also be quantified as to extent, e.g. percent of lung involved. Using this approach allows for determining changes qualitatively and quantitatively over time.

Cost: A reasonable cost <u>per animal</u> for the above is shown below. These estimates are based on my experience and discussions with a pathology lab that I have worked with for ~30 years. I can explain how I came up with these numbers.

Necropsy ---------------------- ---------------	$50.00
Materials -------------------------------------	5.00
Histology H&E (7$/slide)--------------------	14.00
Collagen stain ($20)-----------------	20.00
Pathological exam ($50/slide)---------------	150.00

Path report writing (40 hrs @200/hr) – This is for all of the animals, so until the total number of animals are determined I cannot figure the cost on a per/animal basis.

QA – If this is a GLP study figure add an additional 30% for QA.

Option: If the contract lab cannot come within a reasonable range to the above estimate, you can contract the work to another group of pathologists that would be able to handle this just as well as the contract lab. In this case, the contract lab would do the necropsies, tie-off the lobe for fiber burden and fix the lungs. The lungs would then be sent to the other lab for histology and path exam.

I hope this meets your needs. Give me a call if you have questions.

Sincerely,

Gene McConnell

Proposed Libby Amphibole Inhalation Study Design 06/18/03

Dose Group	Dose Material	Dose Level (mg/m³)	PCME fibers/cc*	Exposure	Recovery	Sacrifice	N**
1a	None	0	0	1	--	1	23
1b	(control)			3	--	3	23
1c				6	--	6	23
1d				12	--	12	23
2a	Libby	10	166	1	--	1	15
2b	Amphibole			3	--	3	15
2c				6	--	6	15
2d				1	11	12	15
2e				3	9	12	15
2f				6	6	12	15
3a	Libby	1	16.6	1	--	1	15
3b	Amphibole			3	--	3	15
3c				6	--	6	15
3d				1	11	12	15
3e				3	9	12	15
3f				6	6	12	15
4a	Libby	0.1	1.66	1	--	1	15
4b	Amphibole			3	--	3	15
4c				6	--	6	15
4d				1	11	12	15
4e				3	9	12	15
4f				6	6	12	15
5a	Libby	0.01	0.166	1	--	1	15
5b	Amphibole			3	--	3	15
5c				6	--	6	15
5d				1	11	12	15
5e				3	9	12	15
5f				6	6	12	15
6a	Reference	10	TBD†	1	--	1	15
6b	Material #1			3	--	3	15
6c				6	--	6	15
6d				1	11	12	15
6e				3	9	12	15
6f				6	6	12	15
7a	Reference	1	TBD	1	--	1	15
7b	Material #1			3	--	3	15
7c				6	--	6	15
7d				1	11	12	15
7e				3	9	12	15
7f				6	6	12	15

Column group "Exposure and Sacrifice Schedule (months)" covers Exposure, Recovery, Sacrifice.

* Estimated number of fibers
** Includes 3 test animals per group designated for whole lung fiber determinations
† TBD, To be determined

Total Numbers of Test Animals

1 month	113
3 months	113
6 months	113
12 months	293
Total	632

OBJECTIVES:

1. Perform an inhalation study in animals that produces reliable dose-response and duration-response data (both short-term and long-term) on the non-cancer effects of Libby amphibole fibers on lung. The purpose of these data is to help evaluate the potential for non-cancer effects in humans exposed to Libby amphiboles by inhalation., and to establish a site-specific RfC.

2. Obtain data on the relative potency of Libby amphiboles compared to one or more well characterized fibrous materials.

PROPOSED STUDY DESIGN

Dose Group	Dose material	Dose level	Exposure Duration	Total N	Sacrifice Times							
					1 wk	2 wk	4 wk	8 wk	16 wk	24 wk	52 wk	78 wk
1	None	--	24 wk	40	5	5	5	5	5	5	5	5
2	LA	A	24 wk	40	5	5	5	5	5	5	5	5
3		B	24 wk	40	5	5	5	5	5	5	5	5
4		C	24 wk	40	5	5	5	5	5	5	5	5
5		D	24 wk	40	5	5	5	5	5	5	5	5
6	Ref Material	B	24 wk	40	5	5	5	5	5	5	5	5
7		D	24 wk	40	5	5	5	5	5	5	5	5

Ben Jordan (cc)
→ Clyde Lossaso

LIBBY AMPHIBOLE INHALATION STUDY

MEETING AGENDA

November 26, 2002

U.S. EPA Conference Center, Timberline Room

8:00 am - 5:00 pm

1. Introductions

2. Review of Study Objectives

3. Test Material Selection and Characterization
   a. Libby Amphibole
   b. Reference Materials → chrysotile; amphibole → amosite? } both for non-; chrysotile preferred

4. Experimental Design
   a. Background information on proposed study/study design
   b. Endpoints
      i. Necropsy
         (1) Preparation of target tissues (Lung, rib cage, diaphragm?)
         (2) Preservation of non-target tissues
      ii. Histopathology (pre-fibrotic and fibrotic lesions)
         (1) Review of scoring; example slides
         (2) Study pathologist selection and training
         (3) Quality assurance issues
      iii. Bronchoalveolar Lavage (BAL) and Pleural Lavage
         (1) How will this data be used by USEPA?
         (2) Who would perform analyses on lavage fluid?
         (3) Cost effectiveness (usefulness of data obtained vs. cost)
      iv. Fiber burden analysis
         (1) Lung
         (2) Other tissue(s)
   c. Dosing and sacrifice schedule
   d. Dose levels of test and reference materials
   e. Number of animals per treatment group

5. Identification of Candidate Study Performers
   a. Type of lab (i.e., contract, academic, or government)
   b. Candidate performers in selected category(s)

6. Proposed Time Line for Study-Related Activities

7. Review of Action Items

Note: There will be a choice of box-lunches provided at 12 noon

PROPOSED STDY DESIGN
11/26/2002

Dose Group	Dose Material	Dose Level (ug/m3)	Exposure and Sacrifice Schedule (mo) Exposure	Recovery	Sacrifice	N
1a	None		2	--	2	6 - 8
1b	(control)		4	--	4	6 - 8
1c			6	--	6	6 - 8
2a	Libby	10,000	2	--	2	6 - 8
2b	Amphibole		2	4	6	6 - 8
2c			4	--	4	6 - 8
2d			4	2	6	6 - 8
2e			6	--	6	6 - 8
3a	Libby	1,000	2	--	2	6 - 8
3b	Amphibole		2	4	6	6 - 8
3c			4	--	4	6 - 8
3d			4	2	6	6 - 8
3e			6	--	6	6 - 8
4a	Libby	100	2	--	2	6 - 8
4b	Amphibole		2	4	6	6 - 8
4c			4	--	4	6 - 8
4d			4	2	6	6 - 8
4e			6	--	6	6 - 8
5a	Amosite	10,000	2	--	2	6 - 8
5b	Reference		2	4	6	6 - 8
5c	Material		4	--	4	6 - 8
5d			4	2	6	6 - 8
5e			6	--	6	6 - 8
6a	Amosite	1,000	2	--	2	6 - 8
6b	Reference		2	4	6	6 - 8
6c	Material		4	--	4	6 - 8
6d			4	2	6	6 - 8
6e			6	--	6	6 - 8

Sacrifies	Min	Max
1 ~~2 mo~~	36	48
3 ~~4 mo~~	36	48
6 mo	96	128
Total	168	224

Table 1 Experimental Design Summary

Parameter	Proposed Study
Study Laboratory	To be determined (TBD)
Test Animal	Male rat; strain TBD
Animal Maintenance and Environment	TBD
Method of Exposure	Nose-only inhalation
Test Material	• Libby Amphibole matched to characteristics of fibers collected during personal sampling in Libby, MT (processing TBD) • USGS will prepare batch test material
Reference Material	• Amosite (source and processing TBD) • Note: Use of chrysotile may be dictated by regulatory considerations?
Fiber Aerosol Analysis	• Mass concentration and uniformity: monitor daily (Study lab) • Fibers/cc: weekly (Use one or more Libby team labs) • Fiber dimensions: TBD; (Libby team lab)
Target Concentrations	• Libby amphibole: 0, 10, 100, 1000, 10,000 ug/m^3 • Reference material: 100, 10,000 ug/m^3
Treatment Group Size	• 8 animals/treatment group; • total = 224 animals
Exposure Schedule	• 6 hours/day, 5 days/week
Duration of Dosing and Recovery Periods	• See Table 1 for details • Treatment groups ▸ 180 days + no recovery ▸ 120 days + 60 day recovery ▸ 60 days +120 day recovery ▸ Interim sacrifices at 60 and 120 days; terminal sacrifice at 180 days
Type and Frequency of Observations	• Clinical signs and morbidity: daily • Body weight: weekly • Food and water consumption?
Necropsy	• Performed on all animals • Complete set of organs/tissues collected • Target organs/tissues processed for histopathological examination and fiber burden analysis • Remaining tissues archived

Parameter	Proposed Study
Endpoints	• Lung histopathology • Lung fiber burden analysis • Thoracic wall/diaphragm fiber burden analysis? • Bronchoaveolar lavage (pending cost estimate and discussion of intended use of prefibrotic data) • Pleural lavage?
Statistical Analysis	TBD
Sentinel Animals	TBD

PROPOSED EXPOSURE LEVELS

EXPOSURE CONCENTRATION

ug/m3	s / cc	PCME/cc	S10/cc	S20/cc
10,000	407	166	78.1	22.4
1,000	40.7	16.6	7.81	2.24
100	4.07	1.66	0.781	0.224
10	0.407	0.166	0.078	0.022
1	0.041	0.017	0.008	0.002

CUMULATIVE EXPOSURE

ug/m3	PCME s/cc*days
10,000	5387
1,000	539
100	54
10	5.4
1	0.5
Libby Resident	25

Estimate, based on

Avg PCM s/cc	0.001
hr/day	24
Days/yr	350
Yrs	70

Mass Requirement

Flow	10	L/min/group
Groups	10	
hr/day	6	
days/wk	5	
wks	26	
Total Vol	4680000	L
	4680	m3
Mass	51999480	ug
	52.0	g

DRAFT – FOR REVIEW BY USEPA ONLY

NON-CANCER EFFECTS IN RATS FROM INHALATION EXPOSURE TO LIBBY AMPHIBOLE

STUDY DESIGN AND RATIONALE

Prepared by
United States Environmental Protection Agency
Region 8

With Technical Assistance from:
Syracuse Research Corporation
999 18th Street
Suite 1975
Denver, CO 80202

June 18, 2003

DRAFT – FOR REVIEW BY USEPA ONLY

TABLE OF CONTENTS

INTRODUCTION

An animal inhalation study on the non-cancer health effects of Libby amphibole has been proposed to support development of a site-specific inhalation reference concentration (RfC) for Libby, MT. This document provides a preliminary overview of the proposed study and the rationale for the selected approach. A detailed description of all study protocols and quality assurance activities will be provided in the Quality Assurance Project Plan (QAPP) pending approval of the proposed study.

BACKGROUND AND PROBLEM IDENTIFICATION

Background

Libby, MT is a community located near an open pit vermiculite mine which began limited operations in the 1920's and was operated on a larger scale by the W.R. Grace Company from approximately 1963 to 1990. Studies at the site have revealed that vermiculite from the mine contains amphibole-type asbestos, and that workers at the mine had an increased risk of developing asbestos-related lung disease. Although the mine has ceased operation, concern exists that historic or continuing releases of asbestos from mine-related materials could be serving as a source of ongoing asbestos exposure and health risk to current and future residents of the area.

Problem Statement

Inhalation exposure to asbestos can cause both cancer and non-cancer effects in the lung. EPA has developed a standard method for estimating cancer risks from inhalation of asbestos fibers, and is currently working to refine and improve that method. However, EPA currently has no established method for estimating the risk of non-cancer effects from inhalation exposure to asbestos, or any way to identify a level of asbestos fibers in air that is below a level of concern for non-cancer effects.

It is sometimes assumed that if the level of asbestos in air is low enough to protect against cancer effects, then the level will also protect against non-cancer effects as well. However, this is not certain. Indeed, a health survey of current residents has shown a much higher incidence of non-cancer asbestos-related disease than asbestos-related cancer. For these reasons, data are needed to establish a site-specific inhalation RfC for amphibole fibers in the community of Libby, MT.

The RfC is an estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of non-cancer deleterious effects during a lifetime. The RfC can be derived from a no observed adverse effect level (NOAEL), lowest observed adverse effect level (LOAEL), or benchmark concentration, with uncertainty factors generally applied to reflect limitations of the data used. There are no existing health effects data on Libby amphibole that are adequate for this purpose.

Intended Use of Data

Data on the non-cancer health effects of Libby amphibole in animals are intended to increase our understanding of the potential health effects in exposed human populations. The data collected in this study will be used to develop a site-specific RfC for exposure to Libby amphibole. Comparison of actual and potential human exposure data collected in Libby, MT to the site-specific RfC will enable identification of routine or special activities potentially associated with increased risk of asbestos-related health effects, and will be useful in making site-specific decisions regarding the need for remedial action to protect human health.

PROJECT DESCRIPTION

Study Objectives

The two main objectives for the Libby Amphibole Inhalation Study are as follows:

1. Establish reliable dose-response and duration-response data for the non-cancer effects of Libby amphibole fibers on the lung of test animals.

2. Obtain data on the relative potency of Libby amphibole compared to one or more well-characterized fibrous materials.

Project Timetable

The project timetable will be developed in conjunction with the study laboratory and study pathologist. This timetable will include specific study activities, their projected start dates, and their anticipated dates of completion. The timetable will be drafted prior to study initiation and included in the QAPP.

MEASUREMENT/DATA QUALITY OBJECTIVES

The general measurement objectives for the Libby Amphibole Inhalation Study include:

1) data representativeness (the physical and chemical properties of the test material will be similar to those of fibers associated with human exposures in Libby, MT)

2) data comparability (study endpoints and test materials have been selected to facilitate comparison to data from existing animal studies)

3) data defensibility (the study will be designed and conducted to ensure that the data collected are scientifically and legally defensible).

Specific measurement/data quality objectives will be identified and justified prior to study initiation using U.S. EPA's seven step Data Quality Objective (DQO) procedure.

STUDY DESIGN

Overview of Proposed Study

Three alternative study designs have been drafted for the purpose of obtaining price quotations from candidate study laboratories. These designs will all provide dose-response and duration-response data for Libby amphibole, but differ in the number of fiber dose groups and number of reference materials utilized, and hence the anticipated cost of performing the study. The preferred study design consists of 632 male Fischer F344 rats randomly divided into 6 fiber dose groups and an air control group. The proposed identity and mass concentrations of test fibers, number of test animals, and the exposure schedule for each treatment group are shown in Table 1.

Under the study design summarized in Table 1, groups of rats (n=15) will be exposed to Libby amphibole mass concentrations of 0.01, 0.1, 1, or 10 mg/m^3 for 1, 3, or 6 months before sacrifice. Additional groups of rats (n=15) will be exposed to the same concentrations for 1, 3, or 6 months and subsequently held for recovery (non-exposure) periods of 11, 9, or 6 months, respectively. Additional test animals (n=15) will be exposed to amosite at concentrations of 1.0 or 10 mg/m^3 using the same exposure-only and exposure-recovery schedules described for Libby amphibole. Control animals (n=23/exposure duration) will be exposed to filtered air for 1, 3, 6, or 12 months. The test animals will be exposed to fiber aerosols or filtered air 6 hours/day for 5 consecutive days/week in flow-past nose-only inhalation chambers. Additional information on animal care, test environment, and test methods are summarized in Table 2.

Choice of Exposure Method and Rationale

Three general exposure routes are available for determining the toxicity of fibers: inhalation, intratracheal administration, and intracavitary administration. The advantages and disadvantages of each route are briefly summarized below.

Nose-Only Inhalation. Test animals are exposed to fibers in air via nose-only inhalation by use of specially designed inhalation chambers. This method of exposure most closely approximates the physiological responses anticipated in human exposures. The chief disadvantages are the need for technical expertise to deliver target doses of well-characterized fibers (e.g., for length and diameter) and the high cost of conducting the exposures.

Intratracheal Instillation. Intratracheal instillation delivers a bolus dose of the fiber to the lung. This method of administration is a popular alternative to inhalation exposure, based on the small quantities of test material required; precise dose delivery; elimination of need for costly and elaborate inhalation exposure equipment and complex technical support; increased safety during test procedures; and reduced cost. Disadvantages of the method include: a dose to the lung that is not representative of an inhalation dose; movement of instilled material to gravity-dependent portion of lung, resulting in a different distribution than obtained via inhalation exposure; and occurrence of high local concentrations that can cause irrelevant pathology that cannot occur with inhalation, via mechanisms not relevant to low-dose inhalation exposure. Use of intratracheal administration is accepted for specific regulatory purposes. For example, the European Commission allows for use of intratracheal instillation biopersistence assays (conducted according to specific protocols) to exonerate synthetic fibers from classification as carcinogens.

Intracavitary Injection. Test animals are given abdominal or pleural injections of a bolus that typically contains 10^6 to 10^9 fibers. Test animals are typically evaluated at the end of their lifespan or when a tumor is identified. Administration by this route is known to produce a high incidence of mesothelioma. Advantages of this route include the relatively low cost of administration and high sensitivity for prediction of carcinogenicity (but see below).. Disadvantages include bypass of all natural defenses; the possibility that a single dose early in life may not produce same responses as lower doses and longer exposure; risk of false positives; and lack of agreement on predictive value of data obtained by this route for lung cancer. Most importantly with regard to the Libby Study, this model is of no use for evaluating non-cancer endpoints. The World Health Organization (WHO, 1992) has concluded that the intraperitoneal injection model should not be used for quantitative risk assessment or for comparing relative hazards posed by different fibers.

Based on the intended use of the data from the Libby amphibole study, nose-only inhalation was selected as the most appropriate and relevant exposure route for evaluating non-cancer endpoints (especially pulmonary fibrosis) in the lungs of test animals.

Procurement and Characterization of Study Materials

Testing of two fiber types is proposed. The proposed fiber types are (a) Libby amphibole, size-selected to simulate the fiber distribution found in Libby, MT personal air samples and (b) amosite asbestos (reference fiber), another durable amphibole fiber which has previously been characterized for health effects in F344 rats.

The Libby amphibole test material used in this study will be derived from a composite bulk sample or asbestos-rich ore collected from the W. R. Grace Mine. This bulk material will be processed to produce a range of asbestos fiber lengths and diameters similar to that observed for air samples in Libby. If necessary, size fractionation techniques may be applied to improve the match in particle size distributions between test material and air samples. This work will be done by the U.S. Geological Survey Laboratory in Lakewood, CO.

The source of the amosite asbestos reference material is to be determined. USEPA is presently researching the availability of UICC amosite used in previous studies conducted by the Agency.

Aerosol Generation and Monitoring

The fiber aerosols will be produced using an aerosol generation system provided by the study laboratory. The aerosol generation system will include a ^{63}Ni charge neutralizer or functional equivalent to reduce the electronic charge on the fibers. The study laboratory will document the ability of the system to generate consistent levels of fibers in air without breaking, grinding, or contamination prior to study initiation. In addition, the study laboratory will demonstrate the ability of the system to deliver uniform levels of aerosol to the ports in the system. Port-to-port variability is anticipated to be less than 10%. Fiber concentrations (expressed as mg/m^3) will be measured at pre-determined frequencies during the pretest and exposure periods. To assure the uniformity of exposure during each 6-hr. exposure period, fiber concentrations will be continuously monitored by the study laboratory using a RAS (GCA Corp.) light scattering monitor or functionally equivalent instrument. Each aerosol will be sampled weekly to determine concentration (expressed both as Phase Contrast Microscopy Equivalent (PCME) fibers/cc and mg/m^3) and for measurement of fiber length and diameter. Samples of airborne fibers will be collected on filters placed in empty animal exposure ports during the animal exposure period. Aerosol sample filters collected by the study laboratory will be sent to the Libby Analytical team for quantitation and fiber analysis. Fibers captured on the sample filters will be processed for quantitation by transmission electron microscopy conducted according to guidelines in ISO 10312.

Selection of Animal Model and Rationale

The study will be conducted using male "specific pathogen free" (SPF) F344 rats purchased from Charles River Breeding Laboratories. Both rats and hamsters were initially considered as possible animal models for the study. The rat was selected because it more closely mimics humans in the development of health effects (i.e., pulmonary fibrosis, lung neoplasms, and mesothelioma) in response to fiber exposure (FSAP, 2001). In addition, hamsterscan be expected to contract a lethal intestinal disease ("wet tail") in chronic studies (> 6 months) because they are not derived to be SPF. Therapeutic intervention for treatment of this disease is always problematic in terms of its potential success and is a potential confounder when interpreting study results (FSAP, 2001). Gender selection was based on a review of asbestos-related health effects including fibrosis (the key endpoint in the proposed study), lung tumors, and mesothelioma. The proposed collection of data using males only is considered acceptable because analysis of a robust database for various types of asbestos and asbestos-related health effects showed that male and female rats are for the most part equally sensitive to induction of fibrosis and neoplasia following exposure to fibers (FSAP, 2001). In cases where a difference in lung tumor incidence was noted, a higher incidence was usually observed in males (FSAP, 2001). The limited data available for mesothelioma response in males and females also do not suggest a marked sex difference (FSAP, 2001). The F344 strain and commercial source were selected to enable the most direct comparison possible to existing asbestos fiber inhalation data.

Animal Maintenance

Weanling male F344 rats will be purchased from a Charles River breeding facility. The rats will be held at the study laboratory until they reach a suitable size (approximately 200-250 g; 10-12 weeks of age) for placement in the restraining tubes of the inhalation exposure chambers. Test animals will be acclimated to the test chambers for one week prior to initiation of fiber exposure. When not being exposed, the test animals will be housed individually in polycarbonate cages containing hardwood bedding in Hazleton 2000 chambers or functional equivalent. The chambers will be housed in rooms operated under negative pressure (-20 mm H_2O) with 12 to 15 air changes/hour. The temperature will be maintained at 22 ± 3°C with relative humidity of 30-70%, on a 12 hour light/dark schedule. The animals will be fed a standard pelleted rat maintenance diet *ad libitum* during non-exposure periods. Filtered water will be supplied in individual bottles *ad libitum* during non-exposure periods.

Dose Selection and Rationale

The proposed target concentrations of Libby amphibole in air are 0.01, 0.1, 1, and 10 mg/m^3. Based on available data from the site, these level are expected to correspond to about 0, 0.17, 1.7, 17 and 170 PCME fibers/cc, respectively. These target concentrations were selected to be representative of short-term and cumulative human exposures that may occur in Libby, MT, as determined from analysis of personal air sampling data collected by U.S. EPA. Testing of fiber mass concentrations at the 0.01 mg/m^3 level will be contingent on the ability to generate and monitor a consistent aerosol at this mass concentration. The target concentrations for the reference fiber will be 1 and 10 mg/m^3. The higher concentration (10 mg/m^3) is the most frequently used target concentration for asbestos fibers in rodent health effects studies and is included to allow comparison of potency across studies. The 1 mg/m^3 concentration is included to ensure that the daily and cumulative exposures in this study are representative of human exposure levels.

Animal Exposure and Monitoring

Modular flow-past, nose-only inhalation chambers will be used to expose the test animals to fiber aerosol. This type of exposure chamber is designed to provide a positive pressure laminar flow individually to each animal. The animals will be contained in polycarbonate restraint tubes designed so that each animal is individually supplied with fresh aerosol and so that exhaled air is immediately exhausted and does not reach other animals in the inhalation chamber. The tail of each test animal will be maintained outside of the restraint tube for regulation of body temperature.

The test animals will be monitored daily for clinical signs, morbidity, and mortality. The animals will be weighed weekly for the first 13 weeks of the study and at least monthly thereafter. Sick or moribund animals will be euthanized by exsanguination following deep anesthesia by intraperitoneal injection of pentobarbital sodium.

Rats used in the study will be obtained from an optimally clean breeding facility to minimize or eliminate potential pathogens that may affect study results. The disease state of the animals on study will be monitored via serology on sera from extra (sentinel) animals in the study room. The sentinel animals will be from the same production source and weanling groups as the study animals and will be maintained under identical environmental conditions. Serum will be collected from randomly selected animals prior to study initiation, at the end of the 6 month exposure period, and an at study termination (12 months). The serum will be processed for determination of antibody titers to the following agents: pneumonia virus of mice, rat corona virus/sialodacryoadenitis virus, Sendai, Toolan's H-1 virus, Kilham rat virus, and *Mycoplasma pulmonis*. The results from serological testing will be recorded in the interim and/or final study reports. In the event that positive results are obtained, care will be taken to distinguish between fiber-related effects/lesions and those attributable to infection.

Study Endpoints and Rationale for Selection

The key toxicological endpoint in this study will be fibrotic change in the lung as determined by histopathologic examination. This endpoint was selected because fibrotic change underlies the development of asbestosis in exposed humans, and hence is a highly relevant basis for establishing an RfC. Asbestosis is diffuse pulmonary intersitial fibrosis characterized by the presence of interstitial collagen deposition. Initial exposure to asbestos fibers results in focal pulmonary interstitial inflammation starting at the terminal bronchiolar junction. With continuing exposure interstitial fibrosis ensues and proceeds peripherally to include the alveolar ducts and adjacent alveoli. As the progression of the disease continues, contraction of the fibrous tissue distorts the architecture of the lung, creating enlarged air spaces enclosed within thick fibrous walls. In humans, difficult or labored breathing (dyspnea) accompanied by coughing is usually the first clinical manifestation of asbestosis (Kumar et al., 1992). The disease may remain static or progress to congestive heart failure, right ventricular enlargement of the heart, and death.

Data on fibrotic change will be supported by data collected on lung weight and gross appearance, fiber burden in the lung, and the physical and chemical characteristics of fibers recovered from the lung. If resources permit, data may also be collected on fiber burden of the diaphragm as indication of fiber migration to the pleura and potential for development of mesothelioma.

Rationale for Duration of Exposure and Recovery Periods

Test animals exposed to different dose levels of Libby amphibole or reference material will be sacrificed after 1, 3, or 6 months of exposure to determine both dose- and duration-dependent patterns of pulmonary pathology (disease). Additional groups will be exposed to Libby amphibole or reference material for 1, 3, or 6 months and subsequently maintained for a non-exposure period of 11, 9, or 6 months, respectively, to evaluate potential recovery from fiber-induced health effects. The lengths of the exposure and recovery periods were selected based on available information regarding the time course of development of fibrotic response to inhaled asbestos, and the expected time-course of potential progression or recovery after exposure. These exposure periods were selected after consultation with Dr. E.E. McConnell of Toxpath, Inc., an expert in the design of fiber inhalation studies and evaluation of fiber-induced pathology.

Post-mortem Examination

The use of consistent methods for postmortem tissue examination and preservation is critical for determination of histopathological lesion severity, fiber burdens, and dose-response relationships. To ensure consistency, necropsies for each group of animals sacrificed will be conducted within 1-2 days of sacrifice. To minimize potential variability associated with the intermittent exposure schedule, each round of sacrifice and necropsy will be conducted on the same day of the week. This schedule will ensure that the test animals have had a consistent fiber exposure prior to postmortem examination.

The general scheme for post-mortem processing of organs and tissues in shown in Figure 1. Test animals will be euthanized by exsanguination following deep anesthesia by intraperitoneal injection of pentobarbital sodium. Use of this method is necessary to avoid introduction of pulmonary lesions during euthanization. The order of necropsy will be controls, low dose, mid doses, and high dose, respectively, to preclude fiber contamination. Extreme care will be taken to ensure that there is no cross-contamination among exposure groups, e.g. Libby fiber with amosite. A complete set of tissues will be collected and fixed in 10% neutral buffered formalin, except for the lungs and diaphragm. The rib cage and nasal cavity will be individually preserved in separate containers of fixative for possible future analysis. The lung will be removed in toto, weighed, and examined under a dissecting scope. The right lung (all four lobes) will be tied off at the level of the bronchus, weighed and frozen at -20°C for subsequent fiber burden determinations. The diaphragm will be removed and preserved separately from the lung for fiber burden analysis (see below). The remaining left lung with trachea attached will be perfused with formalin fixative via the trachea at a pressure of 30 cm H_2O for 2 hours. After fixation, consistently uniform transverse (horizontal) sections (2 mm) of the left lung will be obtained for routine histopathology. In addition, sections will be made from any grossly visible lesions. Replicate samples from each animal will be stained with hematoxylin and eosin (H&E) and Masson-Goldner's trichrome stain for collagen deposition. After removal, a standard 2 cm diameter punch from the middle of the diaphragm will be taken and frozen at -20°C for possible fiber burden analysis. A scientist with training and experience in conducting inhalation studies will travel to the study laboratory to monitor these activities on behalf of USEPA.

Because there are concerns that inhaled fibers may be unevenly distributed between the right and left lungs, three animals per treatment group will be sacrificed at each timepoint for determination of fiber burden in the entire lung. The lung will be removed in toto, weighed, examined under a dissecting scope, and preserved for fiber analysis.

Histopathological Evaluation

The lungs will be examined and classified histologically using the grading scale of McConnell et al. (1984, 1995) as summarized in Table 3. In this grading system, a grade of 1.0 is considered normal; grades 2 and 3 are evidence of focal cellular change and are potentially reversible; and grades 4 through 8 include cellular changes along with increasing degrees of fibrosis. Other types of lesions will be recorded as appropriate. Lung changes reflecting the degree of macrophage infiltration, bronchiolization, microgranuloma formation, and pleural fibrosis will be graded using a scale of 0 to 5 (0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = massive/severe). To ensure reliable and consistent interpretation, all slides will be initially evaluated by a single study pathologist. Dr. E. E. McConnell will provide training and consultation on the use of the grading scale as necessary. Slide evaluations will be verified by one or more independent pathologists familiar with pulmonary lesions associated with exposure to fibers. A detailed description of the verification approach will be provided in the QAPP.

Fiber Burden Analysis

Fiber burden in lung tissue will be determined on samples collected from asbestos-exposed animals at each sacrifice. Background levels of fibers will be determined in several randomly selected control animals at each time point. As described above, the right lung and diaphragm will be removed and frozen (-20°C) for determination of fiber burden. In addition, the entire lung from three animals per treatment group per time point will also be preserved for fiber analysis. The frozen samples will be transferred to a Libby Analytical Team Laboratory for analysis. Each sample will be thawed, rapidly dehydrated with acetone, and dried to constant weight to determine the dry weight of the tissue. The order of processing is important for preventing cross contamination of samples and will be controls, low dose, mid doses, and high doses, respectively. The dry weight of each sample will be measured and recorded. The dried lung and diaphragm (separately) will be minced and plasma ashed in an LFE LTA 504 multiple chamber ashing unit or functionally equivalent apparatus. If necessary, the ash from each lung will be washed with prefiltered household bleach (alkaline hypochlorite solution) at 60°C and pH 9.5-10.5 for 10 minutes to digest residual organic debris; rinsed with filtered deionized water; and passed through a micropore filter. The fiber recovery procedure will be validated with spiked unexposed tissues prior to the start of the experiment as described in Hesterberg et al. (1996). To accomplish this, a known mass of Libby amphibole will be injected into an excised rat lung; the fiber will be recovered using the procedure outlined above; and the mass of the recovered fiber will be measured gravimetrically and compared to the mass injected.

Recovered fibers will be dispersed in distilled water, transferred to an MCE filter, and examined by transmission electron microscopy for number, bivariate dimensions, and mineralogic characteristics. Quantitation of fibers will be conducted according to ISO 10312. Based on this analysis, estimates of lung burden will be expressed as WHO fibers, fibers > 10 µm, and fibers > 20 µm length per milligram dry lung tissue and per gram of wet lung. WHO fibers are defined as those having an aspect ratio greater than or equal to 3:1, length greater than 5 µm, and diameter less than 3 µm. The burden of WHO fibers will be calculated to permit comparison with previous studies. For consistency of evaluation, all fiber analyses will be performed by the same individual over the course of the study.

STUDY LABORATORY REQUIREMENTS

Fiber inhalation studies are among the most expensive and technically challenging toxicology studies to perform. The data generated in the proposed study must be obtained and documented in a manner that is scientifically and legally defensible. To ensure the success of the proposed study, it is essential that the performing laboratory has 1) well-documented prior experience in conducting nose-only fiber inhalation studies; 2) demonstrated capability in conducting GLP-compliant studies; and 3) sufficient space, equipment, and qualified staff to initiate and complete the study in a timely manner. An informal capability review of academic, government, and private research laboratories has been performed to determine which type of laboratory would be best suited to fulfill study requirements. Based on the requirements listed above, a private research or contract laboratory appears to be the best choice to perform this study. Selection of the study laboratory will follow appropriate U.S. EPA contracting procedures.

GOOD LABORATORY PRACTICE

The study will be conducted in accordance with the intent and spirit of U.S. EPA GLP Guidelines (40 CFR Part 792) to the extent permitted by available resources. Any departures from GLP guidelines necessitated by resource limitations or other factors will be documented in the QAPP and/or the interim and final study reports.

QUALITY ASSURANCE

Detailed information on project oversight, data quality objectives, and quality assurance activities will be provided in the QAPP.

DOCUMENTATION AND REPORTING

The format of the study report, schedule for data availability from individual time points, and schedule for preparation of the final report will be determined in consultation with the study laboratory and study pathologist. Appropriate U.S. EPA chain-of-custody procedures will be used for transfer of study materials.

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Table 1 Proposed Libby Amphibole Inhalation Study Design

Dose Group	Dose Material	Dose Level (mg/m^3)	Dose Level PCME fibers/cc*	Exposure and Sacrifice Schedule (months) Exposure	Recovery	Sacrifice	N**
1a	None	0	0	1	--	1	23
1b	(control)			3	--	3	23
1c				6	--	6	23
1d				12	--	12	23
2a	Libby	10	166	1	--	1	15
2b	Amphibole			3	--	3	15
2c				6	--	6	15
2d				1	11	12	15
2e				3	9	12	15
2f				6	6	12	15
3a	Libby	1	16.6	1	--	1	15
3b	Amphibole			3	--	3	15
3c				6	--	6	15
3d				1	11	12	15
3e				3	9	12	15
3f				6	6	12	15
4a	Libby	0.1	1.66	1	--	1	15
4b	Amphibole			3	--	3	15
4c				6	--	6	15
4d				1	11	12	15
4e				3	9	12	15
4f				6	6	12	15
5a	Libby	0.01	0.166	1	--	1	15
5b	Amphibole			3	--	3	15
5c				6	--	6	15
5d				1	11	12	15
5e				3	9	12	15
5f				6	6	12	15
6a	Reference	10	TBD†	1	--	1	15
6b	Material #1			3	--	3	15
6c				6	--	6	15
6d				1	11	12	15

Dose	Dose	Dose Level		Exposure and Sacrifice Schedule (months)			N**
Group	Material	(mg/m³)	PCME fibers/cc*	Exposure	Recovery	Sacrifice	
6e				3	9	12	15
6f				6	6	12	15
7a	Reference	1	TBD	1	--	1	15
7b	Material #1			3	--	3	15
7c				6	--	6	15
7d				1	11	12	15
7e				3	9	12	15
7f				6	6	12	15

* Estimated number of fibers
** Includes 3 test animals per group designated for whole lung fiber determinations
† TBD, To be determined

Total Numbers of Test Animals

1 month	113
3 months	113
6 months	113
12 months	293
Total	632

Table 2 Experimental Design Summary

Parameter	Proposed Study
Study Laboratory	To be determined (TBD)
Test Animal	Male F344 rats (Charles River Breeding Laboratories)
Animal Maintenance and Environment	Cages: polycarbonate Cage filters: TBD Animals per cage: one Bedding: hardwood Temperature: 22 ± 3°C Humidity: 30-70% Room air changes: 15/hour Fluorescent light: 12 hours/day Feed: standard maintenance diet; available *ad libitum* Water: System TBD; available *ad libitum* Method of animal identification: TBD Time held before study: 3 weeks Age at start of study: 10-12 weeks
Method of Exposure	Flow-past, nose-only inhalation
Test Material	Libby Amphibole matched to characteristics of fibers collected in air Libby, MT
Reference Material	Amosite (Source to be determined)
Fiber Aerosol Analysis	Mass concentration and uniformity: monitor daily (Study lab) Fibers/cc: weekly (Libby Analytical Team) Fiber dimensions: weekly (Libby Analytical Team)
Target Concentrations	Libby amphibole: 0.01, 0.1, 1, 10 mg/m^3 Reference material: 1, 10 mg/m^3
Treatment Group Size	15 animals/fiber treatment group; 23 animals/control group (includes 3 animals/treatment group for analysis of total lung fiber burden) total = 632 animals
Exposure Schedule	6 hours/day, 5 days/week
Duration of Dosing and Recovery Periods	See Table 1
Type and Frequency of Observations	Clinical signs and morbidity: daily Body weight: weekly for first 13 weeks and at least monthly thereafter

Parameter	Proposed Study
Necropsy	Performed on all animals Complete set of organs/tissues collected Lung and diaphragm processed for histopathological examination and/or fiber burden analysis Remaining tissues archived
Endpoints	Lung histopathology Lung fiber burden analysis Diaphragm fiber burden analysis (tentative)
Statistical Analysis	TBD
Sentinel Animals	Sentinel animals from same supplier and weanling group as the test animals will be maintained in the animal room. Sera will be collected prior to study initiation, at 6 months, and at12 months; sera will be collected and analyzed for antibody titers (see text)

Table 3 Pathology Grading Scale for Lung Tissue

Pathology	Extent	Grade	Description
Cellular Change	Normal	1	No lesion observed.
	Minimal	2	A few macrophages in the lumen of terminal bronchioles and alveoli.
	Mild	3	Presence of cuboidal epithelium lining the proximal alveoli (epithelialization). No collagen, but reticulin fibers may be present in the interstitium at the bronchiolo-alveolar junction. Luminal macrophages are more conspicuous; mononuclear cells may be found in the interstitium.
Fibrosis	Minimal	4	Minimal collagen deposition at the bronchiolo-alveolar junction; increased bronchiolization; associated mucoid debris suggesting glandular pattern.
	Mild	5	Interlobular linking of lesion described in grades 4 and increased severity of fibrosis.
	Moderate	6	Early consolidation; parenchymal decrease
	Severe	7	Marked fibrosis and consolidation.
		8	Complete obstruction of most airways.

Source: McConnell et al. (1984, 1995)

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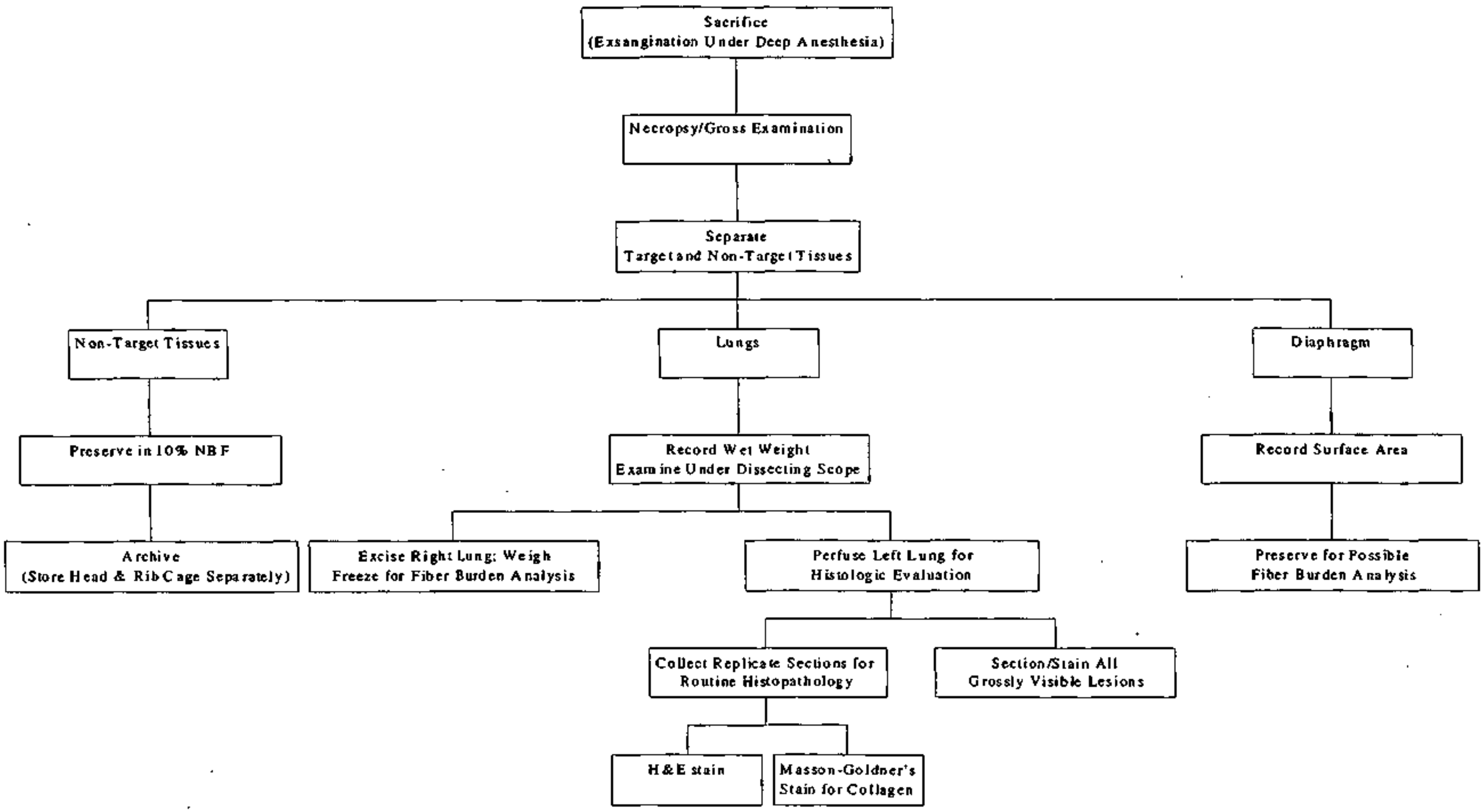
Figure 1 Flowchart for Postmortem Processing of Tissues in the Proposed Libby Amphibole Inhalation Study.



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NON-CANCER EFFECTS IN RATS FROM INHALATION EXPOSURE TO LIBBY AMPHIBOLE

STUDY DESIGN AND RATIONALE

Prepared by
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INTRODUCTION

An animal inhalation study on the non-cancer health effects of Libby amphibole has been proposed to support development of a site-specific inhalation reference concentration (RfC) for Libby, MT. This document provides a preliminary overview of the proposed study and the rationale for the selected approach. A detailed description of all study protocols and quality assurance activities will be provided in the Quality Assurance Project Plan (QAPP) pending approval of the proposed study.

BACKGROUND AND PROBLEM IDENTIFICATION

Background

Libby, MT is a community located near an open pit vermiculite mine which began limited operations in the 1920's and was operated on a larger scale by the W.R. Grace Company from approximately 1963 to 1990. Studies at the site have revealed that vermiculite from the mine contains amphibole-type asbestos, and that workers at the mine had an increased risk of developing asbestos-related lung disease. Although the mine has ceased operation, concern exists that historic or continuing releases of asbestos from mine-related materials could be serving as a source of ongoing asbestos exposure and health risk to current and future residents of the area.

Problem Statement

Inhalation exposure to asbestos can cause both cancer and non-cancer effects in the lung. EPA has developed a standard method for estimating cancer risks from inhalation of asbestos fibers, and is currently working to refine and improve that method. However, EPA currently has no established method for estimating the risk of non-cancer effects from inhalation exposure to asbestos, or any way to identify a level of asbestos fibers in air that is below a level of concern for non-cancer effects.

It is sometimes assumed that if the level of asbestos in air is low enough to protect against cancer effects, then the level will also protect against non-cancer effects as well. However, this is not certain. Indeed, a health survey of current residents has shown a much higher incidence of non-cancer asbestos-related disease than asbestos-related cancer. For these reasons, data are needed to establish a site-specific inhalation RfC for amphibole fibers in the community of Libby, MT.

The RfC is an estimate (with uncertainty spanning perhaps an order of magnitude) of a continuous inhalation exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of non-cancer deleterious effects during a lifetime. The RfC can be derived from a no observed adverse effect level (NOAEL), lowest observed adverse effect level (LOAEL), or benchmark concentration, with uncertainty factors generally applied to reflect limitations of the data used. There are no existing health effects data on Libby amphibole that are adequate for this purpose.

Intended Use of Data

Data on the non-cancer health effects of Libby amphibole in animals are intended to increase our understanding of the potential health effects in exposed human populations. The data collected in this study will be used to develop a site-specific RfC for exposure to Libby amphibole. Comparison of actual and potential human exposure data collected in Libby, MT to the site-specific RfC will enable identification of routine or special activities potentially associated with increased risk of asbestos-related health effects, and will be useful in making site-specific decisions regarding the need for remedial action to protect human health.

PROJECT DESCRIPTION

Study Objectives

The two main objectives for the Libby Amphibole Inhalation Study are as follows:

1. Establish reliable dose-response and duration-response data for the non-cancer effects of Libby amphibole fibers on the lung of test animals.

2. Obtain data on the relative potency of Libby amphibole compared to one or more well-characterized fibrous materials.

Project Timetable

The project timetable will be developed in conjunction with the study laboratory and study pathologist. This timetable will include specific study activities, their projected start dates, and their anticipated dates of completion. The timetable will be drafted prior to study initiation and included in the QAPP.

MEASUREMENT/DATA QUALITY OBJECTIVES

The general measurement objectives for the Libby Amphibole Inhalation Study include:

1) data representativeness (the physical and chemical properties of the test material will be similar to those of fibers associated with human exposures in Libby, MT)

2) data comparability (study endpoints and test materials have been selected to facilitate comparison to data from existing animal studies)

3) data defensibility (the study will be designed and conducted to ensure that the data collected are scientifically and legally defensible).

Specific measurement/data quality objectives will be identified and justified prior to study initiation using U.S. EPA's seven step Data Quality Objective (DQO) procedure.

STUDY DESIGN

Overview of Proposed Study

Three alternative study designs have been drafted for the purpose of obtaining price quotations from candidate study laboratories. These designs will all provide dose-response and duration-response data for Libby amphibole, but differ in the number of fiber dose groups and number of reference materials utilized, and hence the anticipated cost of performing the study. The preferred study design consists of 656 male Fischer F344 rats randomly divided into 8 fiber dose groups and an air control group. The proposed identity and mass concentrations of test fibers, number of test animals, and the exposure schedule for each treatment group are shown in Table 1. Study Design Option 2 also consists of 8 fiber dose groups and an air control group, but incorporates smaller group sizes (Table 2). Study Design Option 3 incorporates the smaller group sizes and tests only one reference material (Table 3). Selection among these designs will be based on price quotations received from the candidate study laboratories.

Under the preferred study design option summarized in Table 1, groups of rats (n=12) will be exposed to Libby amphibole mass concentrations of 0.01, 0.1, 1, or 10 mg/m^3 for 1, 3, or 6 months before sacrifice. Additional groups of rats (n=12) will be exposed to the same concentrations for 1, 3, or 6 months and subsequently held for recovery (non-exposure) periods of 11, 9, or 6 months, respectively. Additional test animals (n=12) will be exposed to amosite or chrysotile asbestos fibers at concentrations of 1.0 or 10 mg/m^3 using the same exposure-only and exposure-recovery schedules described for Libby amphibole. Control animals (n=20/exposure duration) will be exposed to filtered air for 1, 3, 6, or 12 months. The test animals will be exposed to fiber aerosols or filtered air 6 hours/day for 5 consecutive days/week in flow-past nose-only inhalation chambers. Additional information on animal care, test environment, and test methods are summarized in Table 4.

Choice of Exposure Method and Rationale

Three general exposure routes are available for determining the toxicity of fibers: inhalation, intratracheal administration, and intracavitary administration. The advantages and disadvantages of each route are briefly summarized below.

Nose-Only Inhalation. Test animals are exposed to fibers in air via nose-only inhalation by use of specially designed inhalation chambers. This method of exposure most closely approximates the physiological responses anticipated in human exposures. The chief disadvantages are the need for technical expertise to deliver target doses of well-characterized fibers (e.g., for length and diameter) and the high cost of conducting the exposures.

Intratracheal Instillation. Intratracheal instillation delivers a bolus dose of the fiber to the lung. This method of administration is a popular alternative to inhalation exposure, based on the small quantities of test material required; precise dose delivery; elimination of need for costly and elaborate inhalation exposure equipment and complex technical support; increased safety during test procedures; and reduced cost. Disadvantages of the method include: a dose to the lung that is not representative of an inhalation dose; movement of instilled material to gravity-dependent portion of lung, resulting in a different distribution than obtained via inhalation exposure; and occurrence of high local concentrations that can cause irrelevant pathology that cannot occur with inhalation, via mechanisms not relevant to low-dose inhalation exposure. Use of intratracheal administration is accepted for specific regulatory purposes. For example, the European Commission allows for use of intratracheal instillation biopersistence assays (conducted according to specific protocols) to exonerate synthetic fibers from classification as carcinogens.

Intracavitary Injection. Test animals are given abdominal or pleural injections of a bolus that typically contains 10^6 to 10^9 fibers. Test animals are typically evaluated at the end of their lifespan or when a tumor is identified. Administration by this route is known to produce a high incidence of mesothelioma. Advantages of this route include the relatively low cost of administration and high sensitivity for prediction of carcinogenicity (but see below). Disadvantages include bypass of all natural defenses; the possibility that a single dose early in life may not produce same responses as lower doses and longer exposure; risk of false positives; and lack of agreement on predictive value of data obtained by this route for lung cancer. Most importantly with regard to the Libby Study, this model is of no use for evaluating non-cancer endpoints. The World Health Organization (WHO, 1992) has concluded that the intraperitoneal injection model should not be used for quantitative risk assessment or for comparing relative hazards posed by different fibers.

Based on the intended use of the data from the Libby amphibole study, nose-only inhalation was selected as the most appropriate and relevant exposure route for evaluating non-cancer endpoints (especially pulmonary fibrosis) in the lungs of test animals.

Procurement and Characterization of Study Materials

Testing of three fiber types is proposed. The proposed fiber types are (a) Libby amphibole, size-selected to simulate the fiber distribution found in Libby, MT personal air samples; (b) amosite asbestos (reference fiber), another durable amphibole fiber which has previously been characterized for health effects in F344 rats; and (c) chrysotile asbestos (reference fiber), a fiber type that represents over 95% of asbestos usage in the United States and which has been characterized for health effects in F344 rats and Syrian golden hamsters.

The Libby amphibole test material used in this study will be derived from a composite bulk sample or asbestos-rich ore collected from the W. R. Grace Mine. This bulk material will be processed to produce a range of asbestos fiber lengths and diameters similar to that observed for air samples in Libby. If necessary, size fractionation techniques may be applied to improve the match in particle size distributions between test material and air samples. This work will be done by the U.S. Geological Survey Laboratory in Lakewood, CO.

NIEHS intermediate length chrysotile asbestos reference material will be purchased from Research Triangle Institute (RTI), Research Triangle Park, NC. This material has previously been used in oral and inhalation exposure studies conducted by the National Institute of Environmental Health Sciences and detailed information on its chemical and physical properties is available (Campbell et al., 1980). Supplemental analysis of fiber dimensions or chemical properties may be performed by the Libby Analytical Team as deemed necessary. The biological activity of this material via the inhalation route has been assessed in male F344 rats (McConnell et al. 1984; Wagner et al., 1984; Mast et al., 1995) and male Syrian golden hamsters (McConnell et al., 1995).

The source of the amosite asbestos reference material is to be determined. Johns Manville Corporation (Littleton, CO) has been contacted regarding the availability of size-selected amosite reference material used in fiber inhalation studies conducted in F344 rats (e.g., Hesterberg et al., 1998; 1999).

Aerosol Generation and Monitoring

The fiber aerosols will be produced using an aerosol generation system provided by the study laboratory. The aerosol generation system will include a ^{63}Ni charge neutralizer or functional equivalent to reduce the electronic charge on the fibers. The study laboratory will document the ability of the system to generate consistent levels of fibers in air without breaking, grinding, or contamination prior to study initiation. In addition, the study laboratory will demonstrate the ability of the system to deliver uniform levels of aerosol to the ports in the system. Port-to-port variability is anticipated to be less than 10%. Fiber concentrations (expressed as mg/m^3) will be measured at pre-determined frequencies during the pretest and exposure periods. To assure the uniformity of exposure during each 6-hr. exposure period, fiber concentrations will be continuously monitored by the study laboratory using a RAS (GCA Corp.) light scattering monitor or functionally equivalent instrument. Each aerosol will be sampled weekly to determine concentration (expressed both as Phase Contrast Microscopy Equivalent (PCME) fibers/cc and mg/m^3) and for measurement of fiber length and diameter. Samples of airborne fibers will be collected on filters placed in empty animal exposure ports during the animal exposure period. Aerosol sample filters collected by the study laboratory will be sent to the Libby Analytical team for quantitation and fiber analysis. Fibers captured on the sample filters will be processed for quantitation by transmission electron microscopy conducted according to guidelines in ISO 10312.

Selection of Animal Model and Rationale

The study will be conducted using male "specific pathogen free" (SPF) F344 rats purchased from Charles River Breeding Laboratories. Both rats and hamsters were initially considered as possible animal models for the study. The rat was selected because it more closely mimics humans in the development of health effects (i.e., pulmonary fibrosis, lung neoplasms, and mesothelioma) in response to fiber exposure (FSAP, 2001). In addition, hamsterscan be expected to contract a lethal intestinal disease ("wet tail") in chronic studies (> 6 months) because they are not derived to be SPF. Therapeutic intervention for treatment of this disease is always problematic in terms of its potential success and is a potential confounder when interpreting study results (FSAP, 2001). Gender selection was based on a review of asbestos-related health effects including fibrosis (the key endpoint in the proposed study), lung tumors, and mesothelioma. The proposed collection of data using males only is considered acceptable because analysis of a robust database for various types of asbestos and asbestos-related health effects showed that male and female rats are for the most part equally sensitive to induction of fibrosis and neoplasia following exposure to fibers (FSAP, 2001). In cases where a difference in lung tumor incidence was noted, a higher incidence was usually observed in males (FSAP, 2001). The limited data available for mesothelioma response in males and females also do not suggest a marked sex difference (FSAP, 2001). The F344 strain and commercial source were selected to enable the most direct comparison possible to existing asbestos fiber inhalation data.

Animal Maintenance

Weanling male F344 rats will be purchased from a Charles River breeding facility. The rats will be held at the study laboratory until they reach a suitable size (approximately 200-250 g; 10-12 weeks of age) for placement in the restraining tubes of the inhalation exposure chambers. Test animals will be acclimated to the test chambers for one week prior to initiation of fiber exposure. When not being exposed, the test animals will be housed individually in polycarbonate cages containing hardwood bedding in Hazleton 2000 chambers or functional equivalent. The chambers will be housed in rooms operated under negative pressure (-20 mm H_2O) with 12 to 15 air changes/hour. The temperature will be maintained at 22 ± 3°C with relative humidity of 30-70%, on a 12 hour light/dark schedule. The animals will be fed a standard pelleted rat maintenance diet *ad libitum* during non-exposure periods. Filtered water will be supplied in individual bottles *ad libitum* during non-exposure periods.

Dose Selection and Rationale

The proposed target concentrations of Libby amphibole in air are 0.01, 0.1, 1, and 10 mg/m^3. Based on available data from the site, these level are expected to correspond to about 0, 0.17, 1.7, 17 and 170 PCME fibers/cc, respectively. These target concentrations were selected to be representative of short-term and cumulative human exposures that may occur in Libby, MT, as determined from analysis of personal air sampling data collected by U.S. EPA. Testing of fiber mass concentrations at the 0.01 mg/m^3 level will be contingent on the ability to generate and monitor a consistent aerosol at this mass concentration. The target concentrations for the reference fibers will be 1 and 10 mg/m^3. The higher concentration (10 mg/m^3) is the most frequently used target concentration for asbestos fibers in rodent health effects studies and is included to allow comparison of potency across studies. The 1 mg/m^3 concentration is included to ensure that the daily and cumulative exposures in this study are representative of human exposure levels.

Animal Exposure and Monitoring

Modular flow-past, nose-only inhalation chambers will be used to expose the test animals to fiber aerosol. This type of exposure chamber is designed to provide a positive pressure laminar flow individually to each animal. The animals will be contained in polycarbonate restraint tubes designed so that each animal is individually supplied with fresh aerosol and so that exhaled air is immediately exhausted and does not reach other animals in the inhalation chamber. The tail of each test animal will be maintained outside of the restraint tube for regulation of body temperature.

The test animals will be monitored daily for clinical signs, morbidity, and mortality. The animals will be weighed weekly for the first 13 weeks of the study and at least monthly thereafter. Sick or moribund animals will be euthanized by exsanguination following deep anesthesia by intraperitoneal injection of pentobarbital sodium.

Rats used in the study will be obtained from an optimally clean breeding facility to minimize or eliminate potential pathogens that may affect study results. The disease state of the animals on study will be monitored via serology on sera from extra (sentinel) animals in the study room. The sentinel animals will be from the same production source and weanling groups as the study animals and will be maintained under identical environmental conditions. Serum will be collected from randomly selected animals prior to study initiation, at the end of the 6 month exposure period, and an at study termination (12 months). The serum will be processed for determination of antibody titers to the following agents: pneumonia virus of mice, rat corona virus/sialodacryoadenitis virus, Sendai, Toolan's H-1 virus, Kilham rat virus, and *Mycoplasma pulmonis*. The results from serological testing will be recorded in the interim and/or final study reports. In the event that positive results are obtained, care will be taken to distinguish between fiber-related effects/lesions and those attributable to infection.

Study Endpoints and Rationale for Selection

The key toxicological endpoint in this study will be fibrotic change in the lung as determined by histopathologic examination. This endpoint was selected because fibrotic change underlies the development of asbestosis in exposed humans, and hence is a highly relevant basis for establishing an RfC. Asbestosis is diffuse pulmonary intersitial fibrosis characterized by the presence of interstitial collagen deposition. Initial exposure to asbestos fibers results in focal pulmonary interstitial inflammation starting at the terminal bronchiolar junction. With continuing exposure interstitial fibrosis ensues and proceeds peripherally to include the alveolar ducts and adjacent alveoli. As the progression of the disease continues, contraction of the fibrous tissue distorts the architecture of the lung, creating enlarged air spaces enclosed within thick fibrous walls. In humans, difficult or labored breathing (dyspnea) accompanied by coughing is usually the first clinical manifestation of asbestosis (Kumar et al., 1992). The disease may remain static or progress to congestive heart failure, right ventricular enlargement of the heart, and death.

Data on fibrotic change will be supported by data collected on lung weight and gross appearance, fiber burden in the lung, and the physical and chemical characteristics of fibers recovered from the lung. If resources permit, data may also be collected on fiber burden of the diaphragm as indication of fiber migration to the pleura and potential for development of mesothelioma.

Rationale for Duration of Exposure and Recovery Periods

Test animals exposed to different dose levels of Libby amphibole or reference material will be sacrificed after 1, 3, or 6 months of exposure to determine both dose- and duration-dependent patterns of pulmonary pathology (disease). Additional groups will be exposed to Libby amphibole or reference material for 1, 3, or 6 months and subsequently maintained for a non-exposure period of 11, 9, or 6 months, respectively, to evaluate potential recovery from fiber-induced health effects. The lengths of the exposure and recovery periods were selected based on available information regarding the time course of development of fibrotic response to inhaled asbestos, and the expected time-course of potential progression or recovery after exposure. These exposure periods were selected after consultation with Dr. E.E. McConnell of Toxpath, Inc., an expert in the design of fiber inhalation studies and evaluation of fiber-induced pathology.

Post-mortem Examination

The use of consistent methods for postmortem tissue examination and preservation is critical for determination of histopathological lesion severity, fiber burdens, and dose-response relationships. To ensure consistency, necropsies for each group of animals sacrificed will be conducted within 1-2 days of sacrifice. To minimize potential variability associated with the intermittent exposure schedule, each round of sacrifice and necropsy will be conducted on the same day of the week. This schedule will ensure that the test animals have had a consistent fiber exposure prior to postmortem examination.

The general scheme for post-mortem processing of organs and tissues in shown in Figure 1. Test animals will be euthanized by exsanguination following deep anesthesia by intraperitoneal injection of pentobarbital sodium. Use of this method is necessary to avoid introduction of pulmonary lesions during euthanization. The order of necropsy will be controls, low dose, mid doses, and high dose, respectively, to preclude fiber contamination. Extreme care will be taken to ensure that there is no cross-contamination among exposure groups, e.g. Libby fiber with amosite or chrysotile. A complete set of tissues will be collected and fixed in 10% neutral buffered formalin, except for the lungs and diaphragm. The rib cage and nasal cavity will be individually preserved in separate containers of fixative for possible future analysis. The lung will be removed in toto, weighed, and examined under a dissecting scope. The right lung (all four lobes) will be tied off at the level of the bronchus, weighed and frozen at -20°C for subsequent fiber burden determinations. The diaphragm will be removed and preserved separately from the lung for fiber burden analysis (see below). The remaining left lung with trachea attached will be perfused with formalin fixative via the trachea at a pressure of 30 cm H_2O for 2 hours. After fixation, consistently uniform transverse (horizontal) sections (2 mm) of the left lung will be obtained for routine histopathology. In addition, sections will be made from any grossly visible lesions. Replicate samples from each animal will be stained with hematoxylin and eosin (H&E) and Masson-Goldner's trichrome stain for collagen deposition. After removal, a standard 2 cm diameter punch from the middle of the diaphragm will be taken and frozen at -20°C for possible fiber burden analysis. A scientist with training and experience in conducting inhalation studies will travel to the study laboratory to monitor these activities on behalf of EPA.

Histopathological Evaluation

The lungs will be examined and classified histologically using the grading scale of McConnell et al. (1984, 1995) as summarized in Table 4. In this grading system, a grade of 1.0 is considered normal; grades 2 and 3 are evidence of focal cellular change and are potentially reversible; and grades 4 through 8 include cellular changes along with increasing degrees of fibrosis. Other types of lesions will be recorded as appropriate. Lung changes reflecting the degree of macrophage infiltration, bronchiolization, microgranuloma formation, and pleural fibrosis will be graded using a scale of 0 to 5 (0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = massive/severe). To ensure reliable and consistent interpretation, all slides will be initially evaluated by a single study pathologist. Dr. E. E. McConnell will provide training and consultation on the use of the grading scale as necessary. Slide evaluations will be verified by one or more independent pathologists familiar with pulmonary lesions associated with exposure to fibers. A detailed description of the verification approach will be provided in the QAPP.

Fiber Burden Analysis

Fiber burden in lung tissue will be determined on samples collected from asbestos-exposed animals at each sacrifice using the method of Hesterberg et al. (1998). Background levels of fibers will be determined in several randomly selected control animals at each time point. as described above, the right lung and diaphragm will be removed and frozen (-20°C) for determination of fiber burden. The frozen samples will be transferred to a Libby Analytical Team Laboratory for analysis. Each sample will be thawed, rapidly dehydrated with acetone, and dried to constant weight to determine the dry weight of the tissue. The order of processing is important for preventing cross contamination of samples and will be controls, low dose, mid doses, and high doses, respectively. The dry weight of each sample will be measured and recorded. The dried lung and diaphragm (separately) will be minced and plasma ashed in an LFE LTA 504 multiple chamber ashing unit or functionally equivalent apparatus. If necessary, the ash from each lung will be washed with prefiltered household bleach (alkaline hypochlorite solution) at 60°C and pH 9.5-10.5 for 10 minutes to digest residual organic debris; rinsed with filtered deionized water; and passed through a micropore filter. The fiber recovery procedure will be validated with spiked unexposed tissues prior to the start of the experiment as described in Hesterberg et al. (1996). To accomplish this, a known mass of Libby amphibole will be injected into an excised rat lung; the fiber will be recovered using the procedure outlined above; and the mass of the recovered fiber will be measured gravimetrically and compared to the mass injected.

Recovered fibers will be dispersed in distilled water, transferred to an MCE filter, and examined by transmission electron microscopy for number, bivariate dimensions, and mineralogic characteristics. Quantitation of fibers will be conducted according to ISO 10312. Based on this analysis, estimates of lung burden will be expressed as WHO fibers, fibers > 10 µm, and fibers > 20 µm length per milligram dry lung tissue and per gram of wet lung. WHO fibers are defined as those having an aspect ratio greater than or equal to 3:1, length greater than 5 µm, and diameter less than 3 µm. The burden of WHO fibers will be calculated to permit comparison with previous studies. For consistency of evaluation, all fiber analyses will be performed by the same individual over the course of the study.

STUDY LABORATORY REQUIREMENTS

Fiber inhalation studies are among the most expensive and technically challenging toxicology studies to perform. The data generated in the proposed study must be obtained and documented in a manner that is scientifically and legally defensible. To ensure the success of the proposed study, it is essential that the performing laboratory has 1) well-documented prior experience in conducting nose-only fiber inhalation studies; 2) demonstrated capability in conducting GLP-compliant studies; and 3) sufficient space, equipment, and qualified staff to initiate and complete the study in a timely manner. An informal capability review of academic, government, and private research laboratories has been performed to determine which type of laboratory would be best suited to fulfill study requirements. Based on the requirements listed above, a private research or contract laboratory appears to be the best choice to perform this study. Selection of the study laboratory will follow appropriate U.S. EPA contracting procedures.

GOOD LABORATORY PRACTICE

The study will be conducted in accordance with the intent and spirit of U.S. EPA GLP Guidelines (40 CFR Part 792) to the extent permitted by available resources. Any departures from GLP guidelines necessitated by resource limitations or other factors will be documented in the QAPP and/or the interim and final study reports.

QUALITY ASSURANCE

Detailed information on project oversight, data quality objectives, and quality assurance activities will be provided in the QAPP.

DOCUMENTATION AND REPORTING

The format of the study report, schedule for data availability from individual time points, and schedule for preparation of the final report will be determined in consultation with the study laboratory and study pathologist. Appropriate U.S. EPA chain-of-custody procedures will be used for transfer of study materials.

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Table 1 Proposed Libby Amphibole Inhalation Study Design - Option 1 (Preferred)

Dose	Dose	Dose Level		Exposure and Sacrifice Schedule (months)			N
Group	Material	(mg/m³)	PCME fibers/cc*	Exposure	Recovery	Sacrifice	
1a	None	0	0	1	--	1	20
1b	(control)			3	--	3	20
1c				6	--	6	20
1d				12	--	12	20
2a	Libby	10	166	1	--	1	12
2b	Amphibole			3	--	3	12
2c				6	--	6	12
2d				1	11	12	12
2e				3	9	12	12
2f				6	6	12	12
3a	Libby	1	16.6	1	--	1	12
3b	Amphibole			3	--	3	12
3c				6	--	6	12
3d				1	11	12	12
3e				3	9	12	12
3f				6	6	12	12
4a	Libby	0.1	1.66	1	--	1	12
4b	Amphibole			3	--	3	12
4c				6	--	6	12
4d				1	11	12	12
4e				3	9	12	12
4f				6	6	12	12
5a	Libby	0.01	0.166	1	--	1	12
5b	Amphibole			3	--	3	12
5c				6	--	6	12
5d				1	11	12	12
5e				3	9	12	12
5f				6	6	12	12
6a	Reference	10	TBD†	1	--	1	12

Dose Group	Dose Material	Dose Level (mg/m³)	PCME fibers/cc*	Exposure and Sacrifice Schedule (months) Exposure	Recovery	Sacrifice	N
6b	Material #1			3	--	3	12
6c				6	--	6	12
6d				1	11	12	12
6e				3	9	12	12
6f				6	6	12	12
7a	Reference	1	TBD	1	--	1	12
7b	Material #1			3	--	3	12
7c				6	--	6	12
7d				1	11	12	12
7e				3	9	12	12
7f				6	6	12	12
8a	Reference	10	TBD	1	--	1	12
8b	Material #2			3	--	3	12
8c				6	--	6	12
8d				1	11	12	12
8e				3	9	12	12
8f				6	6	12	12
9a	Reference	1	TBD	1	--	1	12
9b	Material #2			3	--	3	12
9c				6	--	6	12
9d				1	11	12	12
9e				3	9	12	12
9f				6	6	12	12

* Estimated number of fibers
† TBD, To be determined

Total Numbers of Test Animals

1 month	116
3 months	116
6 months	116
12 months	308
Total	656

Table 2 Libby Amphibole Inhalation Study - Option 2

Dose Group	Dose Material	Dose Level (mg/m³)	Dose Level PCME fibers/cc	Exposure and Sacrifice Schedule (months) Exposure	Recovery	Sacrifice	N
1a	None	0	0	1	--	1	10
1b	(control)			3	--	3	10
1c				6	--	6	10
1d				12	--	12	10
2a	Libby	10	166	1	--	1	6
2b	Amphibole			3	--	3	6
2c				6	--	6	6
2d				1	11	12	6
2e				3	9	12	6
2f				6	6	12	6
3a	Libby	1	16.6	1	--	1	6
3b	Amphibole			3	--	3	6
3c				6	--	6	6
3d				1	11	12	6
3e				3	9	12	6
3f				6	6	12	6
4a	Libby	0.1	1.66	1	--	1	6
4b	Amphibole			3	--	3	6
4c				6	--	6	6
4d				1	11	12	6
4e				3	9	12	6
4f				6	6	12	6
5a	Libby	0.01	0.166	1	--	1	6
5b	Amphibole			3	--	3	6
5c				6	--	6	6
5d				1	11	12	6
5e				3	9	12	6
5f				6	6	12	6
6a	Reference	10	TBD†	1	--	1	6
6b	Material #1			3	--	3	6
6c				6	--	6	6
6d				1	11	12	6

Dose	Dose	Dose Level		Exposure and Sacrifice Schedule (months)			N
Group	Material	(mg/m³)	PCME fibers/cc	Exposure	Recovery	Sacrifice	
6e				3	9	12	6
6f				6	6	12	6
7a	Reference	1	TBD	1	--	1	6
7b	Material #1			3	--	3	6
7c				6	--	6	6
7d				1	11	12	6
7e				3	9	12	6
7f				6	6	12	6
8a	Reference	10	TBD	1	--	1	6
8b	Material #2			3	--	3	6
8c				6	--	6	6
8d				1	11	12	6
8e				3	9	12	6
8f				6	6	12	6
9a	Reference	1	TBD	1	--	1	6
9b	Material #2			3	--	3	6
9c				6	--	6	6
9d				1	11	12	6
9e				3	9	12	6
9f				6	6	12	6

* Estimated number of fibers
† TBD, To be determined

Total Numbers of Test Animals

1 month	58
3 months	58
6 months	58
12 months	154
Total	328

Table 3 Proposed Study Design - Option 3

Dose Group	Dose Material	Dose Level (mg/m^3)	Dose Level PCME fibers/cc*	Exposure	Recovery	Sacrifice	N
				Exposure and Sacrifice Schedule (months)			
1a	None	0	0	1	--	1	10
1b	(control)			3	--	3	10
1c				6	--	6	10
1d				12	--	12	10
2a	Libby	10	166	1	--	1	6
2b	Amphibole			3	--	3	6
2c				6	--	6	6
2d				1	11	12	6
2e				3	9	12	6
2f				6	6	12	6
3a	Libby	1	16.6	1	--	1	6
3b	Amphibole			3	--	3	6
3c				6	--	6	6
3d				1	11	12	6
3e				3	9	12	6
3f				6	6	12	6
4a	Libby	0.1	1.66	1	--	1	6
4b	Amphibole			3	--	3	6
4c				6	--	6	6
4d				1	11	12	6
4e				3	9	12	6
4f				6	6	12	6
5a	Libby	0.01	0.166	1	--	1	6
5b	Amphibole			3	--	3	6
5c				6	--	6	6
5d				1	11	12	6
5e				3	9	12	6
5f				6	6	12	6
6a	Reference	10	TBD†	1	--	1	6
6b	Material #1			3	--	3	6
6c				6	--	6	6

Dose Group	Dose Material	Dose Level (mg/m³)	Dose Level PCME fibers/cc*	Exposure	Recovery	Sacrifice	N
6d				1	11	12	6
6e				3	9	12	6
6f				6	6	12	6
7a	Reference	1	TBD	1	--	1	6
7b	Material #1			3	--	3	6
7c				6	--	6	6
7d				1	11	12	6
7e				3	9	12	6
7f				6	6	12	6

Exposure and Sacrifice Schedule (months) applies to the Exposure, Recovery and Sacrifice columns.

* Estimated number of fibers
† TBD, To be determined

Total Numbers of Test Animals

1 month	46
3 months	46
6 months	46
12 months	118
Total	256

Table 4 Experimental Design Summary for Preferred Study Option 1

Parameter	Proposed Study
Study Laboratory	To be determined (TBD)
Test Animal	Male F344 rats (Charles River Breeding Laboratories)
Animal Maintenance and Environment	Cages: polycarbonate Cage filters: TBD Animals per cage: one Bedding: hardwood Temperature: 22 ± 3°C Humidity: 30-70% Room air changes: 15/hour Fluorescent light: 12 hours/day Feed: standard maintenance diet; available *ad libitum* Water: System TBD; available *ad libitum* Method of animal identification: TBD Time held before study: 3 weeks Age at start of study: 10-12 weeks
Method of Exposure	Flow-past, nose-only inhalation
Test Material	Libby Amphibole matched to characteristics of fibers collected in air Libby, MT
Reference Material	Amosite (Source to be determined) NIEHS 100% Chrysotile (Research Triangle Institute)
Fiber Aerosol Analysis	Mass concentration and uniformity: monitor daily (Study lab) Fibers/cc: weekly (Libby Analytical Team) Fiber dimensions: weekly (Libby Analytical Team)
Target Concentrations	Libby amphibole: 0.01, 0.1, 1, 10 mg/m^3 Reference material: 1, 10 mg/m^3
Treatment Group Size	12 animals/fiber treatment group; 20 animals/control group total = 526 animals
Exposure Schedule	6 hours/day, 5 days/week
Duration of Dosing and Recovery Periods	See Table 1
Type and Frequency of Observations	Clinical signs and morbidity: daily Body weight: weekly for first 13 weeks and at least monthly thereafter

Parameter	Proposed Study
Necropsy	Performed on all animals Complete set of organs/tissues collected Lung and diaphragm processed for histopathological examination and/or fiber burden analysis Remaining tissues archived
Endpoints	Lung histopathology Lung fiber burden analysis Diaphragm fiber burden analysis (tentative)
Statistical Analysis	TBD
Sentinel Animals	Sentinel animals from same supplier and weanling group as the test animals will be maintained in the animal room. Sera will be collected prior to study initiation, at 6 months, and at12 months; sera will be collected and analyzed for antibody titers (see text)

Table 3 Pathology Grading Scale for Lung Tissue

Pathology	Extent	Grade	Description
Cellular Change	Normal	1	No lesion observed.
	Minimal	2	A few macrophages in the lumen of terminal bronchioles and alveoli.
	Mild	3	Presence of cuboidal epithelium lining the proximal alveoli (epithelialization). No collagen, but reticulin fibers may be present in the interstitium at the bronchiolo-alveolar junction. Luminal macrophages are more conspicuous; mononuclear cells may be found in the interstitium.
Fibrosis	Minimal	4	Minimal collagen deposition at the bronchiolo-alveolar junction; increased bronchiolization; associated mucoid debris suggesting glandular pattern.
	Mild	5	Interlobular linking of lesion described in grades 4 and increased severity of fibrosis.
	Moderate	6	Early consolidation; parenchymal decrease
	Severe	7	Marked fibrosis and consolidation.
		8	Complete obstruction of most airways.

Source: McConnell et al. (1984, 1995)

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Figure 1 Flowchart for Postmortem Processing of Tissues in the Proposed Libby Amphibole Inhalation Study.